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THE REGULATION OF NUCLEOLIN EXPRESSION IN PROSTATE EPITHELIAL CELLS; POSSIBLE INVOLVEMENT OF MYC

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Finally, I would like to dedicate my thesis to my parents. To my father who never had the opportunity to attend college and was proud to see me receive the kind of university education he always yearned for. To my mother who has always placed education above almost all else.

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SUMMARY INTRODUCTION

The focus of the two papers that comprise this thesis is on the expression of Nucleolin in prostate epithelial cells. We examine that expression on two levels, mRNA and protein. Our concern in both cases is not so much the actual mechanism by which the two types of molecules are synthesized, but more the regulatory mechanism(s) that allow for their maintenance. In the case of Nucleolin mRNA, we wished to understand what we think is an aberration in its expression profile in a particular instance, that instance being a prostate metastatic cell line that has been serum-starved and then serum-rescued. Contrary to results obtained by other research groups, our data show that the mRNA level stays constant throughout this particular experiment. We considered *a priori* the different reasons to account for this constancy, and we then conducted experiments to determine which scenario offered the best possible explanation. In the case of Nucleolin protein, we unexpectedly discovered a discrepancy between the expression of the mRNA and that of the protein: despite the downregulation of mRNA after serum starvation, the protein remained at the same level exhibited in asynchronously-growing cells; and despite the rise in the mRNA levels during serum rescue of the cells, the protein level showed no corresponding increase. As in our first paper, we thought of possible explanations for this disparity and then attempted to provide experimental justification for one or more of these explanations.

A breakdown of the following discussion is as follows: 1, a background on Nucleolin will be provided first: although Nucleolin is presented in the Nucleolin literature as a multifunctional factor, its function in cell growth will be emphasized here; with this in mind, the focus will be on its specific activity of ribosome biogenesis; any other activity that is discussed will be presented in light of the possibility that that activity serves a cell-growth purpose; 2, a brief look at Myc will also be provided, with an explanation as to why the oncogene that encodes this protein fits into the picture that we are outlining here; 3, a brief sketch of prostate development; since we used prostate epithelial cells for our work and since their biology may be relevant to the results we derived through our experiments, a concise look at prostate development may help to understand the significance of the results.

Nucleolin Nucleolin is a phosphoprotein that is localized mainly at the nucleolus, where

ribosome biogenesis takes place.

Nucleolin in a ubiquitous protein encoded by a housekeeping gene. The gene consists of 14 exons and 13 introns and is located on chromosome 2 (Srivastava et al., 1990). Exons 2-5 encode the amino-terminus of the human Nucleolin protein, exons 6-13 encode the central domain, and the last exon, 14, encodes the C-terminus. Like genes encoding other proteins participating in ribosome activity, the *nucleolin* gene has introns which themselves possess coding regions for snoRNAs (Nicoloso et al., 1994; Bachellerie et al., 1995; Bachellerie and Cavaillé, 1998). Two species of snoRNAs are produced that belong to the two major families of snoRNAs, the box C/D and the H/ACA families, whose members produce the two types of nucleotide modifications observed in rRNAs (Bachellerie and Cavaillé, 1998; Smith and Steitz, 1997).

Nucleolin possesses modular structure, as implied above. Its amino-terminus is characterized by four domains of acidic amino acids, interspersed within which are regions of basic amino acids. The central domain enables the protein to bind rRNA, with its four RNA-binding domains (RBDs or RRMs [RNA Recognition Motif]'s). The carboxy-terminus possesses repeats that are rich in glycine and arginine.

N-terminus The N-terminus may enable Nucleolin to participate directly in ribosome biogenesis through its interactions with ribosomal proteins (Bouvet et al 1998; Sicard et al 1998). Neither study addressed the particular step(s) in ribosome assembly for which these interactions are necessary. In addition, the N-terminus may allow Nucleolin to participate in a separate activity that might or might not affect ribosome biogenesis. Through an interaction between its N-terminal acidic amino acids and the basic motif of histone 1 (H1) (Ginisty et al 1999), Nucleolin is proposed to bind H1 (Erard et al 1988; Erard et al 1990; Kharrat et al 1991), presumably H1 on linker DNA within rDNA repeats. If H1 is removed, Nucleolin might then interact electrostatically with the exposed linker DNA through its N-terminal basic regions (Ginisty et al 1999).

There are conflicting reports as to whether this particular activity leads to chromosome condensation or results in decondensation (Erard et al 1988; Erard et al 1990; Kharrat et al 1991). The inconsistency in results may be due to differences in methodology. In the study demonstrating chromosome decondensation (Erard et al 1988), the research group mixed purified Nucleolin and chromatin, whose state of

condensation was then measured through circular dichromism. It is possible that one or both preparations contained a contaminating activity that promoted decondensation. Using an *in vitro* nucleosome-assembly assay, Erard *et al* (Erard et al 1988) showed that Nucleolin neither assembled nucleosomes nor disassembled core histones that had already been placed on DNA. Therefore, Nucleolin-related chromosome decondensation likely occurred through its interaction with and possible displacement of H1 from DNA. A separate study discovered that binding between Nucleolin and H1 was maximal when both were phosphorylated (Kharrat et al 1991). A correlation was established between the extent of this binding and the induced DNA condensation (which was determined through dichromatic analysis). Although Erard *et al* (Erard et al 1988) also used phosphorylated Nucleolin and H1 in binding assays, one protein was phosphorylated while the other remained unmodified. Also, non-phosphorylated Nucleolin was used to affect DNA conformation. Chromatin was extracted from logarithmically-growing cells (This point is assumed since the authors do not state otherwise.), in which case phosphorylated H1 likely represented a minor fraction of total DNA-associated H1. Therefore, it was likely the case that non-phosphorylated H1 interacted with unmodified Nucleolin in the mixture that was analyzed through circular dichromism. If the result from the Kharrat *et al* study is correct, that DNA condensation is induced when phosphorylated Nucleolin and phosphorylated H1 are bound together, then the absence of induced DNA condensation that was observed in the Erard *et al* study (Erard et al 1988) may be due to this difference.

Kharrat *et al* used peptides fragments of both Nucleolin (an N-terminal region that contained the Cdc2 phosphorylation sites) and H1 to show interaction between the two, and a homopolymer poly(dA)poly(dT) was employed to assay for the effect of this interaction on its physical state. Therefore it is possible that while a specific portion of Nucleolin can interact with H1 in such a way as to promote DNA condensation, whole Nucleolin may have the opposite effect, possibly as a result of the interactions among the separate activities of its different domains. As an example of this point, while the Nucleolin C-terminus by itself unwound RNA secondary structures (Ghisolfi et al 1992a), a segment of Nucleolin containing this region along with its four RBDs interacted with RNA in such a way that such structures were produced (Ghisolfi et al

1992b). The same group originally making the claim for the ability of Nucleolin to facilitate DNA decondensation found that an N-terminal fragment had no de-condensing effect on deproteinized and sonicated Salmon sperm DNA, while a C-terminal H1 fragment condensed it. In addition, Erard *et al* (Erard et al 1990) found in the same study that the Nucleolin N-terminal fragment augmented condensation by the H1 fragment.

As discussed above, Kharrat *et al* showed that a phosphorylated N-terminal Nucleolin fragment could work with phosphorylated H1 to condense DNA. As the phosphorylation sites on Nucleolin having this effect are sites for Cdc2 (Erard et al 1990; Belenguer et al 1990), which is active during mitosis, it can be concluded that the interaction facilitates chromosome condensation. However, it is just as possible that a separate interaction occurs following mitosis that leads to decondensation during interphase. Whether the same Nucleolin molecule binds linker DNA after displacing H1 or different Nucleolin molecules separately participate in the two binding reactions was not addressed in the works cited. If the interaction actually de-condenses chromosomes, it might be that Nucleolin indirectly functions in rDNA transcription by altering chromosome structure to allow for maximal transcriptional initiation and elongation (Ginisty et al 1999). No work has addressed this particular activity of Nucleolin since the cited studies came out a decade ago. With current focus in cell biology on the effect of chromatin on transcription and replication, it would be interesting to re-address this issue and determine whether or not current technologies would allow for a more precise picture of this specific Nucleolin activity. For instance, the ChIP (chromatin immunoprecipitation) assay may be useful for determining the extent and conditions of Nucleolin binding to endogenous rDNA. As implied above, Nucleolin may promote cell growth indirectly through alterations in chromosome structure that are amenable to rRNA synthesis. If this were true, a fresh examination of the role of Nucleolin in cell growth would be required from the perspective of how these two activities – ribosome biogenesis and chromosome alteration – are coordinated to promote cell growth.

Another possible biological role for which Nucleolin participates in this activity might be the preservation of rDNA genome integrity. This chromosome region is composed of DNA repeats, and so by its nature is subject to both interchromosomal (between homologues) and intrachromosomal recombination. By interacting with H1 to

condense rDNA chromosome, Nucleolin might preserve structural integrity by helping to construct an rDNA chromatin structure that is refractory to such recombination events. In yeast, intrachromosomal recombination between repeats has been shown to result in rDNA excision and subsequently to cell aging (for review see Guarente 1997). Therefore, the cell must utilize regulatory mechanisms that maintain structural integrity of the rDNA region.

Central Domain The central region of Nucleolin contains four RBDs. Although in theory each domain can interact with RNA, it was concluded following initial studies on Nucleolin-rRNA interaction that two of the domains interact with rRNA and that it is the first two that are engaged in this way (Serin et al 1997). The domains separately bind (Serin et al 1997) the opposite sides of a stem-loop structure of rRNA, bringing together the 5' and 3' ends of the recognition sequence and in this way stabilizing the stem-loop structure (Allain et al 2000). The recognition sequence is called the Nucleolin Recognition Element (Ghisolfi-Nieto et al., 1996), and it is located in the internal transcribed spacer (ITS) and the 5' external transcribed spacer (ETS) and the 3' ETS of both the 28S and 18S RNA sequences (Serin et al 1996). UV cross-linking data revealed Nucleolin binding to these various sites (Serin et al 1996). Nucleolin appears to function as a chaperone in this context. Each of the NRE elements is part of a secondary structure whose correct folding in the 5' ETS is essential for a processing event involving the 5' end of pre-rRNA. By stabilizing the stem-loop structure of which the NRE is part, as just described, Nucleolin appears to restrict access to NRE base pairs that might otherwise participate in the formation of alternate secondary structures (Allain et al 2000). Recently it has been discovered that all four RBDs of a single Nucleolin molecule cooperate to bind a single element, known as the Evolutionary Conserved Motif (ECM), which lies downstream to a cleavage site in the 5' region of rRNA (Ginisty et al 2001). To date there is no evidence to indicate that Nucleolin can simultaneously bind two or more RNA species using its different RBDs.

GAR Domain Repeats within the C-terminus that consist of glycine and arginine residues (GAR domain) interact with ribosomal proteins (Bouvet et al 1998). To extract many of these proteins from ribosomes, high salt concentration is required, and this observation is interpreted to mean that such proteins are core ribosomal proteins that are assembled first

in pre-ribosomal proteins (Bouvet et al 1998). In other words, it is thought that the order in which ribosomal proteins are assembled onto pre-ribosomes and their place within the particles can be inferred from the salt concentration that is required to dislodge them from these particles. This observation alone was taken to imply that Nucleolin must participate in early steps of ribosome assembly (Bouvet et al 1998). Further evidence for this model will be provided later.

In addition to its ability to bind ribosomal proteins, the GAR domain can bind RNA non-specifically and with low affinity (Cobianchi et al 1988; Nadler et al 1991; Ghisolfi et al 1992; Kiledjian et al 1992). However, the domain does not appear to influence the RNA-binding specificity or affinity of the RBDs (Ghisolfi et al 1992; Ghisolfi-Nieto et al 1996; Serin et al 1997). The association of the GAR domain with RBDs suggests one possible means of ribosome assembly on the part of Nucleolin and that is to bring together rRNA and ribosomal proteins that are simultaneously bound by a Nucleolin molecule (Bouvet et al 1998). Although this has not been directly demonstrated, two research groups that managed to isolate pre-ribosomal particles containing Nucleolin and ribosomal proteins known to bind Nucleolin found that these interactions were inhibited when the particles were treated with an RNAase (Piñol-Roma 1999; Yanagida et al 2001). This indicated that, in the absence of rRNA, the strength of these interactions was too low to withstand the conditions in which the particles were isolated. However, this data also could mean that rRNA-bound Nucleolin is in contact with the ribosomal proteins.

The C-terminus by itself appears to confer on Nucleolin the capacity to ‘unstack’ bases in RNA secondary structures (Ghisolfi et al 1992a). A rationale for this function (Ginisty et al 1999) is that it may enable Nucleolin to have access to RNA domains that may otherwise be blocked by these structures. It was not specified in this interpretation whether or not the same Nucleolin molecule can both disassemble RNA secondary structures and then go on to interact with uncovered RNA domains. This point is crucial since separate studies (Sipos and Olson 1991; Ghisolfi et al 1992b) found that the central and C-terminal domains together interact with RNA to produce secondary structures. Even if two (or more) Nucleolin molecules cooperate to enable one to bind ‘interior’ RNA domains, it remains to be explained how the C-terminus of one Nucleolin molecule

can disassemble RNA secondary structures, independently of the central region, while both regions of a second molecule can then together facilitate the production of such structures during ribosome assembly.

Data from a recent study support the finding discussed in the previous paragraph that the central and C-terminal regions together can produce RNA secondary structures. When single-stranded DNA fragments that show base-pair complementarity were mixed with a segment encompassing the two Nucleolin domains (p50), the rate with which the strands annealed was significantly enhanced by p50 (Hanakahi et al 2000). Interestingly, the RBD domain alone could not reproduce this activity, and the interpretation was that the binding of the RBDs to the single strand is too stable to allow for the requisite dissociation from the strand to allow it to anneal with its partner. The GAR domain apparently promotes this dissociation. The authors propose a model in which one Nucleolin molecule interacts with an rRNA and then through self-association to a second molecule that is itself bound either to a different region of the rRNA or to another RNA species, like a snoRNA, the two RNA strands are brought together to anneal. To confirm this model, several unexplored features will have to be explained and clarified. For example, does a single Nucleolin molecule bind an RNA species or is homodimerization required? Is homodimerization itself dependent first on RNA binding by one Nucleolin molecule that alters its confirmation such that it can then bind the second Nucleolin molecule? Does homodimerization enhance the ability of the GAR domain to weaken the hold of RBDs on single-stranded polynucleotides? Hanakahi et al may have answered the second question by showing Nucleolin self-binding in a GST assay, although the authors used Nucleolin segments rather than whole Nucleolin to derive this finding. Because Nucleolin is cleaved into smaller fragments (Chen et al 1991; Warrener and Petryshyn 1991; Fang and Yeh 1993) (see discussion below), this approach may not be invalid.

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When it was found that Nucleolin localizes to the nucleolus (Lischwe et al., 1981), the immediate conclusion was that it must participate in ribosome biogenesis. Most of the detected Nucleolin was in the fibrillar component of the nucleolus (Lischwe et al., 1981; Escande et al., 1985), where rRNA processing occurs, while a relatively

lower amount could be located in the outer granular component. None could be detected in the site of rDNA transcription, known as the fibrillar center (the central region of the nucleolus in logarithmically-growing cells). Therefore, the thought was that Nucleolin must participate in ribosome biogenesis through rRNA processing. Further evidence supported this view: 1 the expression of Nucleolin and rRNA seems to be coordinated (Meyuhas et al., 1990); 2 during *Xenopus laevis* embryogenesis, Nucleolin appears before the synthesis of rRNA and ribosomal proteins (Caizergues-Ferrer et al 1989); 3 Nucleolin was found on pre-ribosomal RNA (Herrera and Olson, 1986). The current picture of Nucleolin involvement in rRNA processing is presented in a later section.

As cell growth and the cell cycle are usually coordinated during cell division of the logarithmic phase, ribosomes must be kept in supply and fully functional in dividing cells. Therefore, a factor like Nucleolin would be expected to be required, if not at all periods of the cell cycle, at least during G1. Work on the regulation of Nucleolin mRNA and protein synthesis has not been detailed to the point of pinpointing a signaling pathway(s) that leads to transcriptional control of the *nucleolin* gene and possibly to translational regulation of its mRNA. With the exception of a study that indicates that the androgen receptor may transactivate the *nucleolin* gene, no work has detailed the DNA- and RNA-binding factors that directly participate in these regulatory mechanisms. In the androgen receptor study, direct action of the receptor on the *nucleolin* gene was implied, but not directly demonstrated through standard assays of DNA binding and transcriptional activation (Tawfic et al 1994). For the most part correlative evidence has been presented to confirm that Nucleolin as promoter of cell growth is employed as cells begin to enter the proliferative phase. As an example, in one study examining liver regeneration, as remaining hepatocytes started to divide following a hepatectomy, the *nucleolin* gene appeared to be transactivated (Ohmori et al 1990). When peripheral blood mononuclear cells were stimulated to grow, the Nucleolin mRNA was stabilized, giving the appearance of a gradual rise in its synthesis; the Nucleolin protein in turn rose to indicate translation of the newly-stabilized mRNA (Westmark and Malter 2001). Transcriptional upregulation of the *nucleolin* gene was observed when serum-starved HeLa cells were replenished with serum (Konishi et al 1995). One research group investigating the possibility that oligonucleotides may function through a non-antisense

mechanism found that the G-rich DNA strands that they introduced into cells bound Nucleolin protein (Bates et al 1999; Xu et al 2001). These cells were inhibited from proliferating, and the research group established a correlation between the degree of inhibition and the amount of bound Nucleolin.

Some of the most extensive and consistent work relating to the biology of Nucleolin in its participation in cell growth has concentrated on its phosphorylation and the cell-cycle dependence of this modification. Nucleolin is phosphorylated during interphase by casein kinase II (Belenguer et al 1989; Bouche et al 1994; Bonnet et al 1996) and is again phosphorylated, at different sites, during mitosis by $p34^{cdc2}$ (Belenguer et al 1990; Zhu et al 1999). Nucleolin is also a target of the cdk3-Cyclin D4, cdk3-Cyclin D6, cdk2-Cyclin E and cdk2-Cyclin A kinase complexes (Sarcevic et al 1997). The significance of these last four instances of phosphorylation has not been explored since their discovery. However, targeting by cdk2-Cyclin A is telling because this hints at an unprecedented role for Nucleolin during G2. The mitosis-specific phosphorylation event (on threonine's in the N-terminus [Belenguer et al 1990]) is difficult to understand because of conflicting interpretations regarding its purpose. During *Xenopus* development Nucleolin with its threonine residues phosphorylated is translocated into the cytosol (Schwab and Dreyer, 1997). The *S pombe* homologue of Nucleolin, Gar2p, is phosphorylated by $p34^{cdc2}$ (Gulli et al., 1997), and yet a non-phosphorylatable Gar2p was found to be sufficient to rescue a *gar2*-null strain (Gulli et al., 1997). Therefore, in this particular organism mitosis-specific phosphorylation of Nucleolin is dispensable. The *S cerevisiae* homologue, Nsr1p, lacks $p34^{cdc2}$-specific sites altogether, and so one interpretation that has been offered to account for this finding is that this particular phosphorylation is unnecessary in a cell like *S cerevisiae* in which no nuclear division accompanies cytokinesis (Ginisty et al 1999). The authors do not elaborate on this idea, but the inference that is drawn here for the purpose of this discussion is that in cells that undergo karyokinesis, phosphorylated threonine on Nucleolin might provide binding sites for other proteins that localize Nucleolin to its proper location during mitosis as the nucleolus dissipates and the nuclear envelope breaks down. Whether or not this localization function is related to the phosphorylation-dependent translocation of Nucleolin to the cytosol (Schwab and Dreyer, 1997) has not been investigated. On the

one hand, phosphorylated threonine appears to enter Nucleolin into the export process that shuttles it to the cytosol. This process may superficially seem related to Nucleolin localization during mitosis in *S cerevisiae*: since there is no nuclear breakdown during *S cerevisiae* mitosis, Nucleolin remains in place, and so a modification that directs it to a new location seems to be irrelevant. On the other hand, because Nucleolin freely enters the cytosol when the nuclear envelope disappears in eukaryotes that follow karyokinesis as they divide, a localization-specific modification event seems at first glance to be unnecessary in this case as well. Hence, it cannot be that phosphorylated threonine results only in the shuttling of Nucleolin across the nuclear membrane: the modification must direct it to a specific site, whether in the nucleus or in the cytosol. This particular localization might be mediated by factors that specifically bind phosphorylated Nucleolin. However, modification of Nucleolin may not be required, as it was shown in a recent study (Zhu et al 1999) that Nucleolin with all its phosphorylation sites mutated could localize during mitosis as wild-type Nucleolin did. The method employed was transient transfection of constructs into mammalian cell lines, both the mutated Nucleolin gene and the wild-type gene as a control. Given data that Nucleolin can self-associate (Hanakahi et al 2000), it is possible that exogenous mutated Nucleolin was moved to the correct cellular spots by endogenous phosphorylated Nucleolin to which it was bound. More work will have to be conducted in this area, especially on the particular activity that is conferred upon Nucleolin through its $p34^{cdc2}$-mediated phosphorylation. Unless such modification serves only as a localizing device, it must also have an effect(s) on Nucleolin conformation and in this way on its function(s).

One possible purpose of $p34^{cdc2}$-mediated phosphorylation of Nucleolin may be to enhance its helicase activity (Tuteja et al 1995). In fact Nucleolin phosphorylation by $p34^{cdc2}$ and by Casein kinase II (see below) acted in additive fashion to stimulate it. In this study Nucleolin was found to able to unwind DNA-DNA, DNA-RNA, and RNA-RNA duplexes in a 5' to 3' direction.

Casein kinase II (CKII) is sufficient to activate rDNA transcription, at least in adult bovine aortic endothelial (ABAE) cells (Belenguer et al 1989). In this study rRNA synthesis, along with Nucleolin phosphorylation state, dropped when cells reached confluency. rDNA in nuclei that were isolated from confluent cells began transcription

when CKII was added to the nuclei. Commencement of rRNA synthesis correlated with increased Nucleolin phosphorylation. This finding led to the question of what factor activates CKII, and in a follow-up study (Bouche et al 1994) the same group found that FGF-2 not only performed this function but also could do so through direct contact with CKII. It had already been discovered that FGF-2 can enter the cell and translocate to the nucleus (Baldin et al 1990). When FGF-2 was added to nuclei isolated from G1-arrested cells, Nucleolin phosphorylation state and rDNA transcription concomitantly increased. FGF-2 was then found to bind the regulatory subunit of CKII, which stimulated its kinase activity towards Nucleolin (Bonnet et al 1996).

Another question that needs to be addressed is whether or not $p34^{cdc2}$-mediated phosphorylation of Nucleolin is dependent on prior phosphorylation by CKII.

An area of focus on Nucleolin activity that is potentially confusing concerns the possibility that the proteolysis of Nucleolin that is observed under certain conditions may be a requisite step in its activity. Chen *et al* (Chen et al 1991) found that Nucleolin was cleaved in a solubilized extract that was derived from mitogen-stimulated T cells and that was subsequently incubated at 37^0 C for an increasing amount of time (and then analyzed through SDS-PAGE and blotting). This experimental set-up appeared to mimic the resting cell state since extract made from quiescent (non-activated) cells showed Nucleolin degradation. That the cleavage event was self-catalyzed was established through the demonstration that isolated Nucleolin in an SDS gel was subject to the same cleavage after the gel was incubated at 37^0 C (and the SDS was removed from the gel). In a related experiment, the research group purified Nucleolin from an exponentially-growing leukemia line, and after incubating the protein at 37^0 C, found degradation to have occurred. When purified Nucleolin was pre-incubated with the extract from which it had been derived, before the 37^0 C incubation, degradation was prevented. Therefore, the conclusion was that exponentially-growing cells must have an activity that negates the self-cleaving ability of Nucleolin. This activity must be absent in quiescent cells. In a follow-up study (Fang and Yeh 1993), the catalytic center for cleavage was found to reside in the Nucleolin segment encompassing its four RBDs and the GAR domain.

Therefore, it would appear that quiescent cells have degraded and presumably inactive Nucleolin. Yet in a separate study (Warrener and Petryshyn 1991), proteolysis

of Nucleolin appeared to result from CKII-dependent phosphorylation of Nucleolin. As discussed previously, CKII activity induces rDNA transcription leading to cell growth. Through some pathway, phosphorylated Nucleolin assists in this process. If Nucleolin phosphorylation directly were to cause its cleavage, then one or more of the resulting fragments (From the data in the Warrener and Petryshyn study, one fragment stands out, of size p 30 kDa.) would have to facilitate rRNA synthesis. If so, a Nucleolin fragment can itself be functional in a specific activity. This conclusion would not be surprising and can be predicted from the modular structure of Nucleolin, which indicates that each region has functional independence from other regions. However, in light of the data discussed in the previous paragraph, the data by Warrener and Petryshyn is potentially confusing. A simple way to reconcile the results is to conclude that the two cleavage events are different. In one event (as described by the Yeh group) cleavage is self-catalyzed and is prevented by a factor that is either expressed or activated while cells actively proliferate. In the other event cleavage deliberately activates Nucleolin and is induced by prior phosphorylation of whole protein. However, in the Warrener and Petryshyn study nuclear extract was derived from confluents cells, and therefore Nucleolin in this instance came from resting cells. Their SDS gels show Nucleolin of full length. It is possible that Nucleolin self-cleavage is actually activated in resting T cells (the Yeh group study) and that Nucleolin fragments perform activities that are both cell-type (T cell) and condition-specific (resting state). Therefore, as cells proliferate, it could be that what actually becomes activated is an inhibitor of the factor that induces Nucleolin self-cleavage. Nucleolin degradation that could be observed in the SDS gel in which Nucleolin was isolated could be an artifact of the system. Nucleolin self-cleavage may be a ‘default state’ under non-physiological conditions, which the hypothetical activator catalyzes under a specific condition. It should also be noted that in the Warrener and Petryshyn study, CKII activity was not directly demonstrated, but inferred because phosphorylation and proteolysis of Nucleolin was induced by spermine, an activator of CKII. Also, use of heparin, an inhibitor of CKII activity, prevented Nucleolin degradation. Interestingly, the kinase assay using the nuclear extract revealed baseline Nucleolin phosphorylation even in the presence of heparin. This points to the activity of a separate kinase that can phosphorylate Nucleolin and that is not affected by

heparin. Since the cells were quiescent, that kinase cannot be the mitotic $p34^{cdc2}$. Because whole nuclear extract was employed, the possibility should also be considered that spermine might activate a separate kinase that is active during quiescence. Its phosphorylation of Nucleolin might lead to its degradation.

rDNA Transcription The exact role, if any, that Nucleolin has in rDNA transcription has been speculative, until recently (Roger et al 2002). One model for rDNA transcription incorporates the data that was discussed in the previous paragraph: full-length Nucleolin is believed to inhibit rDNA transcription, while the Nucleolin fragment that results from phosphorylation-dependent cleavage allows for elongation while remaining bound to the pre-RNA transcript as it is being synthesized (Ginisty et al 1999). In an *in vitro* assay, Nucleolin bound single-stranded DNA with higher affinity than with double-stranded DNA, and its binding was directed towards the non-transcribed spacer of rDNA upstream to the transcription-initiation site (Olson e al 1983). If this data accurately reflects *in vivo* initiation of rDNA transcription, it indicates that Nucleolin binds DNA that is already in an open conformation that allows accessibility to initiation factors. Nucleolin might function in an earlier step to allow for this open conformation (see 'N-terminus' earlier in this discussion). Its binding to rDNA may inhibit initiation or elongation, and there is data to support this idea. In one study rRNA synthesis was enhanced after an inhibitory anti-Nucleolin antibody was injected into *Chironomus tentans* salivary-gland cells (Egyhazi et al 1988). In a separate study whole Nucleolin inhibited *in vitro* rDNA transcription (Bouche et al 1984). When isolated nucleoli were the 'template' for a nuclear run-off assay, rRNA synthesis proceeded even after Nucleolin was added to the *in vitro* mixture (Bouche et al 1984). However, the inclusion of a protease inhibitor (Leupeptin) led to inhibition of rDNA transcription. The interpretation was that the isolated nucleoli contained the putative Nucleolin-specific protease that cleaved the added Nucleolin to allow transcription to progress. Leupeptin negated that protease, resulting in accumulation of full-length Nucleolin that inhibited transcription. Based on this data, the model of rDNA transcription (Bouche et al 1984) presents full-length Nucleolin as an inhibitor of rDNA transcription: the N-terminus interacts with pol I to prevent elongation (Ginisty et al 1999); upon phosphorylation by CKII, Nucleolin is cleaved and the segment encompassing the central and GAR domains is released; this

segment in turn binds or remains bound to the growing pre-rRNA transcript to process and modify it as it is being transcribed. How the N-terminus as part of whole Nucleolin inhibits pol I but as a fragment has no such effect, has not been elucidated. There has been no discussion in the Nucleolin field as to how Nucleolin self-cleavage fits into this model. The implication of the model is that full-length Nucleolin remains bound to rDNA while cells are quiescent and that activation of rDNA transcription when cells are stimulated to grow and proliferate results from cleavage of Nucleolin by a Leupeptin-susceptible protease. It is possible that a separate pool of Nucleolin exists from which Nucleolin fragments are produced following self-cleavage; these fragments might then go on to participate in activities that are distinct from rDNA transcription and that may or may not promote cell growth.

A study from this year (Roger et al 2002) shows that Nucleolin-mediated repression of rDNA transcription does not involve its binding the growing rRNA transcript. According to the model described immediately above, it is the simultaneous binding of Nucleolin to the growing transcript, through its central domain, and to pol I or another protein, through its N-terminus, that inhibits pol I-mediated elongation of rDNA transcription. The repression that Roger *et al* found was in fact dependent on Nucleolin binding either to the pol I promoter or to the initiation complex itself. However, interaction between Nucleolin and the growing nascent transcript was unnecessary, and the nature of the transcript itself was irrelevant. The main effect that excess Nucleolin had was to reduce the number of actively transcribing pol I complexes rather than the number of rDNA genes that could be engaged. Future work will have to elucidate the mechanism of this repression.

rRNA Processing The primary rRNA transcript containing the 28S (25S in yeast), 5.8S, and 18S rRNA units is cleaved in the 5' external transcribed spacer (ETS) as rDNA transcription occurs (Ginisty et al 1998). Nucleolin is directly involved in this process. The exact details are still unknown. In the current picture Nucleolin functions by binding the ECM (evolutionary conserved motif) and then acting as a scaffold-like factor for other proteins like U3 snoRNP, which is essential for processing to occur. RNA binding itself may modify the conformation of Nucleolin so that it can interact with other proteins involved in the processing reaction. The RNA-dependence of these specific protein-

protein interactions could explain why they do not appear to occur until Nucleolin is bound to the ECM. ECM binding is essential but not sufficient for processing, and the other requisite activities appear to be performed by those participants that interact with Nucleolin directly or indirectly and that themselves subsequently bind separate regions of rRNA. The exact involvement of the Nucleolin Recognition Sequences (NREs) is difficult to assess. *Xenopus* Nucleolin can correctly process rRNA in the *in vitro* processing assay. However, it is unable to bind NREs. The majority of these binding elements reside in variable (sequence variable) regions of the 28S rRNA (Serin et al 1996) that have been found to be essential for ribosome biogenesis. It was discussed earlier that by stabilizing the NRE stem-loop structure, Nucleolin may allow for the proper assembly of secondary structures that contain the NREs. It is also known that the ECM must be in a single-stranded conformation in order for the processing reaction to proceed. Therefore, it is possible that by binding the NREs, Nucleolin creates a local structural environment that maintains the ECM as a single strand. Nucleolin then may bind the ECM to initiate formation of the protein complex that then goes on to cleave rRNA in the 5' ETS. Nucleolin that was added to the *in vitro* processing assay increased the amount of the cleavage product, suggesting that its binding to pre-rRNA is rate-limiting for this particular processing. However, the exact significance of this observation is difficult to understand since the role of the processing reaction itself in ribosome biogenesis is unknown at this point (Ginisty et al 1998).

The N-terminus is necessary for the 5' ETS processing reaction. In the *in vitro* processing reaction p50 (Nucleolin segment containing its RBDs and the GAR domain) had an inhibitory effect. This finding is worth noting since in the model discussed earlier relating the role that cleavage of Nucleolin may play in rDNA transcription, it is p50 that binds the growing pre-rRNA transcript after being released from the N-terminal segment, following Nucleolin phosphorylation by CKII. Further work must address the true significance of the proteolysis of Nucleolin and what role, if any, Nucleolin fragments have in cell growth.

Two research groups managed to isolate premature ribosomal complexes containing Nucleolin (Piñol-Roma 1999; Yanagida et al 2001). Both groups used the same strategy, targeting Nucleolin for isolation and determining its interacting partners,

both ribosomal and non-ribosomal. The advantage of this strategy over the more traditional approach of centrifugal sedimentation is that it is less likely to purify a contaminating particle. The stability of all the complexes isolated in the work by Yanagida *et al* hinged on Nucleolin binding to rRNA since an RNA strand containing NRE could break them apart (presumably by titrating out Nucleolin). Consistent with past results (Bouvet et al 1998), the data from the Yanagida *et al* study revealed the presence of core ribosomal proteins (see 'C-terminus' in earlier part of discussion) in the Nucleolin-containing complexes. Piñol-Roma found that complexes isolated from interphase and mitotic cells had similar protein composition, indicating that complex formation could occur despite cessation of RNA synthesis (during mitosis). In addition, rRNA contained in the isolated complexes ranged from precursor to mature 18S and 28S rRNAs. This finding suggests not only that Nucleolin binds pre-rRNA, as demonstrated already in other studies examining rDNA transcription (Roger et al 2002) and rRNA processing (Ginisty et al 1998), but also that Nucleolin remains bound to rRNA during ribosome-subunit maturation.

Nucleolin Interacting Non-ribosomal Proteins and mRNAs Nucleolin can interact with DNA outside the rDNA region, suggesting a real extra-nucleolar function in the nucleus. Its participation in repressing transcription of the □*1 acid glycoprotein* (AGP) gene (Yang et al 1994) is difficult to relate to cell growth, although as a response factor to tissue injury, AGP might possibly be viewed as an impediment to recovery-phase cell growth and for this reason might have to have its mRNA downregulated by a growth-promoting factor like Nucleolin. A component of the LR-1 transcription complex (Brys and Maizels 1994; Hanakahi et al 1997), Nucleolin activates the *myc* gene (Brys and Maizels 1994), and this particular activity is easier to reconcile with growth promotion as Myc has been documented to induce cell growth (Johnston et al 1999).

By itself or in association with hnRNP D (to form the LR-1 complex), Nucleolin binds G-rich DNA, including the immunoglobulin heavy chain switch regions (Hanakahi et al 1999; Dempsey et al 1999). Through a specific process involving this interaction, Nucleolin may facilitate switch recombination in B cells. Since antibody expression is essential to B-cell development and growth, it may not be surprising to see Nucleolin as a participant. Given the affinity of Nucleolin for sequences enriched for guanine

nucleotides, the 'recruitment' of Nucleolin to participate in this activity may have been an evolutionary maneuver to coordinate a particular type of DNA-binding specificity with growth promotion.

Nucleolin can also interact with mRNAs, such as the Amyloid Protein Precursor mRNA (Zaidi and Malter 1995), Il-2 mRNA (Chen et al 2000), and FMR1 (directly involved in Fragile X syndrome) mRNA (Ceman et al 1999). As Il-2 is required for T-cell activation (with growth and proliferation), the role that Nucleolin plays to stabilize its mRNA may be another example of the coordination of an activity that directly affects cell growth, such as ribosome biogenesis, with a cell type-specific activity whose fulfillment is indirectly related to, but necessary for, cell growth.

Nucleolin binding to Topoisomerase I (Edwards et al 2000) may be consistent with growth promotion in that Topo I, as a regulator of DNA structure, might directly enhance cell growth, or indirectly by helping to create the preconditions for growth. Nucleolin interacts with the transcription factor Myb to negate its activity (Ying et al 2000), and because Myb stimulates proliferation, interference of its activity by Nucleolin is surprising. More investigation will be required here to shed light on this curious finding. The nucleolus has been proposed to be a heat sensor, using Nucleolin to inhibit replication as a response to heat shock (Wang et al 2001). In this study Nucleolin prevented Replication Protein A from facilitating SV40 DNA replication. Resumption of cell growth and proliferation after a disturbance to the cell may depend on such a response, and so, again, a growth promoter like Nucleolin may be temporarily re-deployed to take care of matters before cells can then proceed through the cell cycle.

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A genetic approach to Nucleolin function has not been extensively pursued. Neither knockout organism (like mice or *Drosophila*) nor a transgenic organism has been constructed, and no mammalian cell line exists with the endogenous *nucleolin* gene deleted or amplified. Nucleolin orthologues in *S cerevisiae* and *S pombe* exist and are known as Nsr1p and Gar2p, respectively. The genes encoding both were knocked out in the respective yeast species with similar results (Girard et al 1992; Gulli et al 1995; Kondo and Inouye 1992). The one difference was that while the *S cerevisiae* knock-out strain was viable, the *S pombe* strain produced few survivors (Gulli *et al* isolated four

haploid strains that managed to grow, but predictably, at a very slow rate). Gar2p was able to rescue the *nsr*-null strain in its ability to produce both ribosomal subunits (Kondo and Inouye 1992). Both knockout types were defective in a specific processing event involving the 35S rRNA precursor from which mature 18S, 5.8S, and 25S rRNA species arise. Because of this defect, 18S rRNA was conspicuously absent, and subsequently the 40S subunit was assembled in neither of the two knockout types. In addition, methylation of the 18S rRNA was absent in the *nsr*-null strain. A separate Nucleolin orthologue in *S cerevisiae* known as Gar1p lacks RBDs, and knockout of the gene encoding this protein results in lethality. There appeared to be no rRNA processing at all in this knockout strain, leaving the 35S primary rRNA transcript as the sole rRNA species in the knockout cells. The fact that the *nsr*-knockout strain survived but was slow-growing indicates that Gar1p and Nsr1p together may act, possibly cooperatively, in ribosome biogenesis. Since Gar1p lacks RBDs, it may interact with Nsr1p, exploiting its RNA binding capacity, to place itself in proximity of the 35S transcript during the initial processing events that culminate in release of 18S, 5.8S, and 25S transcripts. In the absence of Nsr1p, Gar1p may be able to bind rRNA through its two Gar domains; however, this binding is probably much less efficient. It is curious that only Gar1p can initiate the processing reactions using the 35S transcript as template: since Nsr1p has a Gar domain, it should in theory be able to perform the same task; that Gar1p has two such domains may be the basis for an explanation. It will be just as interesting to determine whether or not Nsr1p and Gar1p can bind with one another, either in the absence of RNA or only after one or both factors first are RNA bound.

Interestingly, hamster Nucleolin could not rescue the ribosome deficiency of the *nsr*-null strain (Xue et al., 1993). Mammalian Nucleolin directly participates in processing of the nascent pre-rRNA transcript (Ginisty et al 1998), but that processing event may be the earliest one to occur and may be unimpeded in the yeast species as other rRNA transcripts besides 18S rRNA appeared to be produced normally. The processing event that leads specifically to 18S rRNA appears to be one that yeast Nucleolin is uniquely suited to carry out. The conclusion from these studies is that while rRNA is synthesized in yeast in the absence of the Nucleolin orthologues, rRNA processing fails to occur properly, with subsequent depletion of 18S rRNA, and therefore

of the 40S subunit, in the case when Nsr1p and Gar2p are absent, and with the lack of processing of the 35S transcript in *cerevisiae* when Gar1p is absent.

Myc The protein encoded by the *myc* oncogene is known to promote cell proliferation and cell growth and to induce apoptosis under certain conditions (for review see (Grandori et al 2000). The bulk of data that exists concerning its role in cell differentiation indicates that it is prohibitory to this process. It is not known with certainty whether this interference with differentiation is due to the proposed ability of Myc to inhibit cell-cycle exit or results from direct Myc action on signaling pathways leading to differentiation. Myc is a transcription factor belonging to the bHLH family. It possesses both a DNA-binding domain and a transactivation domain. By interacting with another bHLH protein Max, Myc can bind its target DNA site that is called the E-box element. The Myc-Max complex is believed to activate a certain set of genes and to inhibit another set. The determination of such target genes has occupied a large part of Myc studies. The current list of target genes is somewhat controversial, as it has been difficult to confirm the actual target-gene status of many of these genes. For example, in a rodent fibroblast cell line in which the *myc* gene was deleted through homologous recombination, most of the Myc target genes were shown to be unaffected (Mateyak et al 1997). However, recent work has shed some light on the curious finding (Eisenman 2001) that the transactivation capacity of Myc is weak. The Myc-Max complex interacts with a complex that possesses HAT activity and with the Snf5 orthologue, INI1, of a chromatin-remodeling complex (McMahon et al 1998; Cheng et al 1999). ChIP analysis of certain Myc target genes revealed that H4 within their promoters is indeed acetylated following Myc binding (Bouchard et al 2001; Frank et al 2001). It was also shown that, during terminal differentiation when the Myc-Max complex is displaced at target-gene sites by the inhibitory Max-Max complex, the same H4's are de-acetylated and the transcriptional activity previously exhibited by these genes is silenced (Bouchard et al 2001; Xu et al 2001).

Current work on Myc is focused on its apparent sufficiency to induce cell growth (when it is ectopically expressed) (Neufeld et al 1998), indicating that it may induce a series of activities that collectively promote cell growth. Such a series may be in the form of a cascade of gene-expression sets. With this in mind, researchers investigating

target genes of Myc have studied genes encoding ribosomal factors, non-ribosomal factors that are involved in ribosome biogenesis, and factors directly participating in translation. Five research groups have independently found that Myc is capable of activating the *nucleolin* gene (Greasley et al 2000; Coller et al 2000; Kim et al 2000; Boon et al 2001; Schumacher et al 1999; Frank et al 2001). The basic approach taken in all the cited studies is similar: ectopic *myc* was overexpressed, and Nucleolin mRNA synthesis was detected through a standard RNA assay. None of the studies attempted to determine whether or not Nucleolin protein also increased.

While this approach shows that Myc *can* activate the *nucleolin* gene, it does not address the issue of whether or not endogenous Myc actually does transactivate the gene, and if it does so, under what specific intracellular conditions. It is improbable that Myc activates all its bona-fide target genes at all times; it is more likely that its distribution is carefully regulated such that in one particular condition (cell quiescence), it upregulates (and downregulates) a subset while in another (cell proliferation), it affects a different subset of genes. Another consideration is the possible ramification of E-box predominance throughout the genome: an increase in Myc levels may lead to accidental binding to sites that Myc normally does not interact with (Cole and McMahon 1999). Transactivation of such genes may nevertheless be worth examining since this may be a common occurrence in Myc-associated cancers in which Myc levels are higher than normal (Cole and McMahon 1999). Because an increase in Myc level led to enhanced cell growth (Kim et al 2000; Boon et al 2001; Schumacher et al 1999), it appears to be the case that Myc targets a rate-limiting step in cell growth. If one of the activities of Nucleolin is this step, then an increased amount of Nucleolin may accelerate that step. Excepting its helicase capacity, the other documented activities of Nucleolin appear to require it as a structural factor. So a higher Nucleolin level might be required in those instances for a faster pace. A sub-stoichiometric Nucleolin level should be sufficient for its helicase activity. Proof that Myc induces cell growth through Nucleolin would require a clear demonstration that the Nucleolin molecules that are synthesized following ectopic Myc synthesis and action at the *nucleolin* gene participate in an activity that can be increased in its rate; to complete this proof, it would have to be clearly shown that an acceleration of that activity directly leads to increased cell growth.

Prostate Development Prostate development begins during embryogenesis as a bud of epithelial cells that arises from the urogenital sinus, composed of endodermal cells, that is itself derived from the hindgut (for review see Abate-Shen and Shen 2000). The bud develops into ductal structures that grow extensively with elaborating branches into the surrounding mesenchyme. Any given prostate duct branches out into ductules that end in glands. A prostate gland consists of three types of epithelial cells that are surrounded by a basement membrane, which separates the cells from surrounding stromal cells and blood vessels. The three epithelial cell types are luminal secretory cells, basal cells that form a continuous layer between the basement membrane and the luminal cells, and neuroendocrine cells that are interspersed in the basal-cell layer.

Results from experiments involving tissue recombination (Cunha et al 1987; Cunha 1996; Hayward et al 1997) show that both epithelial and stromal cells are required for prostate development. Androgens support early prostate development through the mesenchyme, as prostate fails to form when mesenchymal cells that are defective in the androgen receptor are combined with normal epithelial cells. Later in development androgens stimulate epithelial luminal cells to make and to secrete secretory proteins.

The relationship between prostate epithelial cells and stromal cells is relevant to cancer. Although prostate neoplasms arise from epithelial cells, results from tissue recombination experiments suggest that aberrant growth-factor signaling from stromal cells is crucial for neoplastic development and cancer progression (Hayward et al 1997; Olumi et al 1999).

As is the case with colorectal cancer, prostate cancer appears to progress through a series of histopathologically-identifiable steps that can be correlated with specific genetic events (for review, see Abate-Shen and Shen 2000). Prostatic Intraepithial Neoplasia (PIN) is considered to be a true precursor to prostate cancer, displaying the characteristics of dysplasia and disruption of normal tissue architecture. Invasive carcinoma arises as a clone in which the requisite genetic event has occurred, such as loss of the tumor suppressors *PTEN*, *Rb*, and *p53*. The carcinoma cells break through the basement-membrane capsule. The progression from carcinoma to metastasis is considered to represent a shift from androgen dependence on the part of the carcinoma cells to androgen independence. Metastatic cells usually spread to bone.

PAPER 1

ABSTRACT

Nucleolin is a nucleolar phosphoprotein that has been documented to play a direct role in ribosome biogenesis and other cell activities, some of which are not directly involved in cell growth. Its expression at the transcriptional level is regulated in a cell type- and stimulus-specific manner. Contrary to past results, our data indicate that the *nucleolin* gene is transactivated neither by the androgen receptor that is bound by an agonist of dihydrotestosterone nor by the bHLH protein Myc that is encoded by the proto-oncogene *myc*. In several of our cell lines, the level of Nucleolin mRNA rose in response to serum stimulation of previously serum-starved cells. However, in one specific cell line the level was neither lowered after a serum-deprivation period nor raised during serum stimulation. In attempting to understand the basis for this constancy in the level of Nucleolin mRNA, we determined that the mRNA is not aberrantly stabilized in the cell line displaying the aberrant expression level of Nucleolin mRNA. Because Myc protein also was also held at a constant level in this particular cell line, we considered the possibility that it was maintaining a constant rate of transcription at the *nucleolin* locus. However, we found that endogenous Myc does not transactivate expression of the *nucleolin* gene. We consider the possible ramifications of constancy in the expression, at the mRNA level, of a gene like *nucleolin*.

INTRODUCTION

Nucleolin is a nucleolar phosphoprotein that has been implicated in ribosome biogenesis. To date there is no indication that it is a component of the functional ribosome. However, the available evidence points to a role in facilitating the assembly of ribosome subunits. It is currently unknown which subunit it is whose assembly that mammalian Nucleolin aids. Work with its yeast orthologues, Nsr1p in *S cerevisiae* and Gar2p in *S pombe*, suggests that the subunit in question may be the 40S subunit (Girard et al 1992; Gulli et al 1995; Kondo and Inouye 1992). Nucleolin has modular structure, with N- and C-terminal domains that can interact with ribosomal proteins (Bouvet et al 1998; Sicard et al 1998). Its central domain binds rRNA using two of its four RNA-binding domains (RBD or RRN [RNA Recognition Motif]) (Serin et al 1997). Nucleolin has been shown to bind rRNA through a specific interaction between RBDs 1 and 2 and a stem/loop structure in rRNA known as the Nucleolin Recognition Element (NRE) (Ghisolfi-Nieto et al 1996; Serin et al 1997). All four RBDs are used in the binding of a separate rRNA domain known as the evolutionary conserved motif (ECM) (Ginisty et al 2001). The protein may be able to interact simultaneously with rRNA and ribosomal proteins. In this way it may act as a scaffold-like factor within the granular component of the nucleolus where ribosome-subunit assembly is thought to occur. By simultaneously binding the two components of the ribosome subunit, rRNA and ribosomal proteins, a Nucleolin molecule may orient these components in such a way that they can properly interact to form a ribosomal subunit. In addition Nucleolin participates in rRNA processing. It functions directly in cleaving the 5' end of the rRNA primary transcript and indirectly in modifying rRNA nucleotides (Ginisty et al 1998; Nicoloso et al 1994; Bachellerie et al 1995; Bachellerie and Cavaillé, 1998; Serin et al 1996).

The Nucleolin gene can be transcriptionally activated following cell-growth stimulation that is provided by serum (Konishi et al 1995). The protein is phosphorylated after such stimulation of cells and following androgen-stimulation of prostate cells in culture (Belenguer et al 1989; Bouche et al 1994; Bonnet et al 1996; Suzuki et al 1985). There is evidence to indicate that Nucleolin has self-proteolytic activity that appears to be on when cells are in a quiescent state (Chen et al 1991), such as that which occurs during a period of serum deprivation. Whether this self-proteolysis depends on a de-

phosphorylation event has not been exactly determined. The serum-induced phosphorylation itself leads to a specific cleavage of Nucleolin that has been proposed to separate the N-terminus from the remaining portion of the protein (Warrener and Petryshyn 1991). *In vitro* data indicates that this cleavage allows for PolI-mediated transcription of the rDNA locus to continue after initiation (Bouche et al 1994; Ginisty et al 1999).

There has been little work devoted to examining the fate of the Nucleolin RNA following its synthesis. No outline exists to sketch out the specific pathway that the Nucleolin hnRNA undergoes as it is spliced and exported into the cytosol. No data is available concerning any further processing that the Nucleolin mRNA may be subject to and possible factors with which it may interact. Also, Nucleolin mRNA stability under different cell-culture conditions has not been determined.

The protein encoded by the *myc* oncogene is known to promote cell proliferation and cell growth and to induce apoptosis under certain conditions (for review see (Grandori et al 2000). The bulk of data that exists concerning its role in cell differentiation indicates that it is prohibitory to this process. It is not known with certainty whether this interference with differentiation is due to the proposed ability of Myc to inhibit cell-cycle exit or results from direct Myc action on signaling pathways leading to differentiation. Myc is a transcription factor belonging to the bHLH family. It possesses both a DNA-binding domain and a transactivation domain. By interacting with another bHLH protein Max, Myc can bind its target DNA site that is called the E-box element. The Myc-Max complex is believed to activate a certain set of genes and to inhibit another set. The determination of such target genes has occupied a large part of Myc studies. The current list of target genes is somewhat controversial, as it has been difficult to confirm the actual target-gene status of many of these genes. For example, in a rodent fibroblast cell line in which the *myc* gene was deleted through homologous recombination, most of the Myc target genes were shown to be unaffected (Mateyak et al 1997). However, recent work has shed some light on the curious finding (Eisenman 2001) that the transactivation capacity of Myc is weak. The Myc-Max complex interacts with a complex that possesses HAT activity and with the Snf5 orthologue, INI1, of a chromatin-remodeling complex (McMahon et al 1998; Cheng et al 1999). ChIP analysis

of certain Myc target genes revealed that histone 4 (H4) within their promoters is indeed acetylated following Myc binding (Bouchard et al 2001; Frank et al 2001). It was also shown that, during terminal differentiation when the Myc-Max complex is displaced at target-gene sites by the inhibitory Max-Max complex, the same H4's are de-acetylated and the transcriptional activity previously exhibited by these genes is silenced (Bouchard et al 2001; Xu et al 2001).

Four different research groups have independently found that the *nucleolin* gene is transcriptionally activated by Myc (Greasley et al 2000; Coller et al 2000; Kim et al 2000; Boon et al 2001; Schumacher et al 1999; Frank et al 2001).

The *nucleolin* gene appears to be activated directly by the androgen-receptor complex when prostate cells have been stimulated by androgen (Tawfic et al 1994; Grad et al 1999). The androgen receptor belongs to the steroid-thyroid family of nuclear hormone receptors (see Feldman and Feldman 2001 for review). Like other nuclear hormone receptors it is held at bay within the cytosol by heat-shock protein (HSP). However, upon binding its ligand dihydrotestosterone, it undergoes a conformation change that frees it from HSP and allows it to homodimerize. This results in its phosphorylation and its translocation into the nucleus where it can then activate target genes, such as the gene encoding PSA (Prostate Specific Antigen). The data showing the transcriptional susceptibility of the *nucleolin* gene to androgen was derived through the use of castrated rodents. Castration resulted in depletion of testosterone, and such rodents were found to have an undetectable level of Nucleolin protein in prostate nuclei. Administration of dihydrotestosterone (DHT) resulted in immediate synthesis of Nucleolin. There is also the possibility that Myc may cooperate with activated AR to affect AR target genes. Myc was shown to augment AR transcriptional activity, and given its ability to transactivate the *nucleolin* gene, the possibility has to be considered that together Myc and AR may stimulate expression from the *nucleolin* gene in prostate epithelial cells.

Our original intention was to confirm the previous finding that the *nucleolin* gene can be transcriptionally activated by the androgen-androgen receptor complex. However, our result contradicts the aforementioned data. We also derived the unexpected finding of constancy in the Nucleolin mRNA level in LnCp cells. LnCp cells represent a cell line

that was derived from a prostate metastasis. Unlike the majority of other prostate cell lines, it has retained the androgen receptor and remains androgen sensitive as a result. We found using a LnCp-based culture system that not only is the *nucleolin* gene insensitive to androgen, but also the Nucleolin mRNA remains constant even after a period of serum depravation. This finding also contradicts previous results that show that the gene is transcriptionally silent during cell quiescence and can be activated again upon serum rescue of cells. Our paper investigates the basis for the constancy in the mRNA level. We pursued the following possibilities: 1, the *nucleolin* gene is amplified in LnCp cells, the extra copy being outside the normal regulatory control of the gene; 2, the *nucleolin* gene remains transcriptionally active even in the absence of growth-stimulating factors in serum; 3, in the absence of transcription, the mRNA remains as a result of aberrant stabilization; 4, as the level of Myc protein is also constant in our LnCp cells, the constancy in the Nucleolin mRNA level may be due to this constancy in the Myc level, given the past finding that Myc directly transactivates the *nucleolin* gene.

MATERIALS & METHODS

Cells and reagents

The following cell lines from our collection of prostate cell lines were kindly provided by Dr. Shiv Srivastava of USUHS: LnCp, and PC3. We acquired one of our normal prostate cell lines, the Crl cell line, from the ATCC (CRL-221). The pair of prostate cell lines (Npt & Cpt), which was derived from the same prostate, was generously provided to us by Dr. Guy Tillinghast, formerly of Dr. Kathleen Kelly's lab in the NCI. Dr. Tillinghast also provided us with the rodent fibroblast cell lines, Rat1a and Myc-/- (the latter, our designation). Ingredients for cell-culture media are the following: 1 for LnCp cells, RPMI-1640 (Invitrogen/Life Technologies [I/LT]) with 10% FCS ([I/LT]) and 1% Pen/Strep ([I/LT]); 2 for PC3 cells, DMEM F12 Ham (Sigma) with 10% FCS and 1% Pen/Strep; 3 for Crl cells, Defined Keratinocyte SFM (I/LT) with 10% FCS and 1% Pen/Strep; 4 for Npt and Cpt cells, Defined Keratinocyte SFM with 10% FCS, 1% Pen/Strep, 1% Hepes ([I/LT]), 1% Glutamine ([I/LT]), and 1 ml growth supplement for Defined Keratinocyte SFM (supplied with culture medium); 5 for Rat1a and Myc-/- cells, DMEM ([I/LT]) with 10% FCS, 1% Pen/Strep, and 1% Sodium Pyruvate ([I/LT]).

The cDNA probe we used to assay for PSA mRNA expression was generously provided to us by Dr. Takegawa Segawa of USUHS. The *myc* probe consisted of the entirety of the cDNA from the *myc* gene and was supplied to us by Dr. Michael Keuhl of the NCI. The cDNA had been subcloned into an expression cassette, and we cleaved this vector with EcoR1 to release the entire cDNA. The cDNA was then gel-purified following the Wizard DNA purification system (Promega). For our *nucleolin* probe we used an expression vector into which the *nucleolin* genomic DNA had already been subcloned. Using the PCR (Perkin Elmer) technique, we amplified the amino terminus. We then gel-purified this fragment using a similar Wizard kit designed for the purification of PCR fragments.

For Western blotting we employed the following antibodies (Ab): for Nucleolin, one Ab was purchased from Santa Cruz, and the other from MBL (Medical and Biological Laboratories, Co., LTD.); for Myc, the Ab was purchased from Santa Cruz; for Actin, the Ab was purchased from Santa

Cruz. The secondary Ab was purchased from Amersham Life Science.

Assay for mRNA

RNA was extracted from cells and purified using Trizol (I/LT). Briefly, for a 100-mm culture dish, 1 ml of Trizol was added straight to cells after culture medium was removed and discarded. After lysing of cells was complete, the 1 ml was transferred to an eppendorf tube. The Trizol protocol that was provided with product was then followed. Briefly, the protocol involves lysing of cells through the Trizol reagent, followed by chloroform-based extraction of and isopropanol-mediated precipitation of RNA. RNA was stored in RNAase-free dH_2O at -70^0 C when it was required for assay work.

The RNA gel consisted of the following ingredients in the following amounts (for a 200 ml gel): 1) 2 g of agarose (FMC Bioproducts); 2) 20 ml 10x running buffer [200 mM MOPS (Sigma), 50 mM NaAcetate (Quality Biological, Inc.), 10 mM EDTA (Quality Biological, Inc.)]; 3) 3.6 ml Formaldehyde (Mallinckrodt). Resolution of RNA was achieved using 200 V, with the running time varying from 2 to 4 hours. Blotting was conducted using nitrocellulose paper (Schleicher Scheull) and transfer was left to occur overnight. After transfer RNA was cross-linked to the nitrocellulose paper using a UV cross-linker (Stratagene). Hybridization occurred in a hybridization cylinder. The blot paper was sufficiently soaked in hybridization buffer (178 ml dH_2O, Dextran [100 g in 200 ml dH_2O] (Amersham Pharmecia), 400 ml Formamide (Fluka Chemika), 200 ml 20xSSC (Roche), 10 ml 2 M Tris, pH7.4 (I/LT), 10 ml 100x Denhardt's (Research Genetics), 2 ml Salmon Sperm DNA (I/LT)) before the radioactive probe was added to the cylinder.

The probe was prepared by using Ready-To-Go DNA Labeling Beads (Amersham Pharmecia): briefly, after mixing 100 nM of DNA with dH_2O to bring the total volume up to 45 µl, ^{32}P-CTP (Amersham Biosciences) was added (5 µl) along with a DNA bead; the probe was then incubated at 37^0 C – the incubation time depended on the amount of labeling that was desired. Labeled probe was purified through EtOH precipitation: labeled DNA was phenol-chloroform extracted, after which it was incubated in dry ice with NaAcetate and 100% EtOH; the mixture was then microcentrifuged, the supernatant was discarded, and then the DNA pellet suspended in 100 µl of dH_2O. The labeled probe was then added to the following mixture: 1) 1.3 ml of dH_2O; 2) 200 µl Salmon Sperm; 3)

50 μl of 10N NaOH (Mallinckrodt). After probe addition, the following ingredients were then added, to complete probe preparation: 1) 140 μl of 2M Tris, pH7.5 (I/LT); 2) 500 μl of 1N HCl (Mallinckrodt). This entire mixture was then added to the cylinder containing the blot paper that had been soaked through with hybridization buffer. The cylinder containing the blot was left overnight at 42^0 C.

After probe annealing was allowed to occur, the blot was washed in the following way: 1) 3 washes at room temperature using 0.1% SDS (I/LT)/2x SSC at 15 min/wash; 2) 1 wash at 65^0 C using 0.1% SDS/0.1x SSC for 15 min. The blot was then wrapped in saran wrap and left exposed to Kodak film at -70^0 C, to allow for autoradiography to occur.

Assay for protein

For protein extraction cells were first harvested in culture through culture-dish scraping in ice-cold PBS (scraping done on ice; cell-culture medium first removed before addition of PBS). Following transfer to a 15-ml conical tube, the cells were then centrifuged (5 min at 1200 rpm). After discarding the supernatant, the cell pellet was broken up and cells suspended in ice-cold PBS. The cells were then transferred to an eppendorf, which in turn was spun down to obtain a cell pellet (5 min at 13 Krpm [microcentrifuge]). Cells were lysed in buffer consisting of the following ingredients: 1) 0.5% 2M Tris; 2) 3% 5M NaCl (Digene); 3) 1% 500 mM EDTA; 4) 1% Triton (Sigma); 5) 10% Glycerol (I/LT); 6) 2 mM Vanadate (Sigma). Cells were left on ice for 5 min to allow cell lysis to reach completion, at which point the released material was spun down to remove cell debris (5 min at 13 Krpm). The supernatant was transferred to a fresh eppendorf, which was then stored at -70^0 C until it was required for protein resolution through an SDS gel.

Protein gels were made using the following ingredients: for the stacking gel (4.5%) – 1) 0.9 ml Acrylamide/bis (BioRad) (30%/0.8%); 2) 1.5 ml Buffer solution 1x; 3) 0.06 ml 10% APS (BioRad); 4) 0.01 ml 100% Temed (BioRad); 5) 3.6 ml H_2O/ for the resolving gel (12%) – 1) 3.6 ml Acrylamide/bis (30%/0.8%); 2) 2.25 ml Buffer 1x; 3) 0.063 ml 10% APS; 4) 0.012 ml 100% Temed; 5) 3.15 ml H_2O. The buffer for the stacking gel consisted of the following: 1) 0.5 M Tris; 2) 0.4% SDS; adjusted to pH of 6.9. The ingredients for the buffer used in the resolving gel: 1) 1.5 M Tris; 2) 0.4% SDS; adjusted to pH 8.8. Separation of proteins in the gel was achieved at a voltage of 200 V, using a

gel apparatus from (BioRad). Proteins were then transferred to nitrocellulose paper using the same voltage and a transfer apparatus from the same company. The transfer buffer consisted of the following: transfer buffer: (1 L) 100 ml of Methyl EtOH (Fisher Scientific), 3.2 g Tris (I/LT), 14.4 g Glycine (ICN Biomedicals, Inc.). The blot was blocked in a solution of milk (5% milk in PBS with 1% BSA [Sigma]) for 1 hr (or in some cases overnight). Following an overnight exposure to the primary Ab, the blot was washed 4x in PBS/Tween-20 (Sigma) (0.1%). Exposure to the secondary Ab was for 30 min, followed by 3 washes in the PBS/Tween-20 solution. The blot was soaked in Pierce SuperSignal solution, briefly dried and wrapped in Saran Wrap, and then exposed to Kodak film for different times at room temperature.

Figures and Legends to Paper1

Fig 1 The nucleolin gene is not transcriptionally responsive to androgen. LnCp cells were placed in culture containing RPMI and 10% FCS. 48 hr later, the cells were washed 2x with PBS to remove any trace of androgen that exists in FCS. RPMI was then added containing FCS from which androgen had been depleted (charcoal-treated FCS). The cells were left in incubation for 10 days. On the 11^{th} day, the medium was replaced with fresh RPMI containing the same charcoal-treated serum in which an androgen agonist (R1881) was included. R1881 was added at a concentration of 10^{-10} M. The cells were then placed back in incubation for the indicated time points. For each time point a particular culture was taken out and lysed for RNA extraction. The RNA samples were run through a formaldehyde-agarose gel and then transferred to a nitrocellulose blot, which was then probed with radioactively-labeled oligonucleotides (first the oligonucleotide from a psa cDNA, and then after stripping a nucleolin-specific probe, and finally, after a third stripping a GADPH-specific probe. (a) The mRNA for PSA was assayed for as a control to show that this culture-based system works. The PSA mRNA level dropped as expected in the absence of activity by androgen receptor (indicated by lane 1, denoted '0 hr'). The androgen agonist was effective, as indicated by the steady rise of PSA mRNA following agonist 'rescue' of the cells. (b) The level of Nucleolin mRNA at the same time points (c) 28S and 18S rRNA levels as our loading control (d) GAPDH mRNA level through the time course of agonist rescue as another, visual, loading control. 'Nuc' denotes Nucleolin, and 'AgR' denotes agonist rescue.

Fig 1

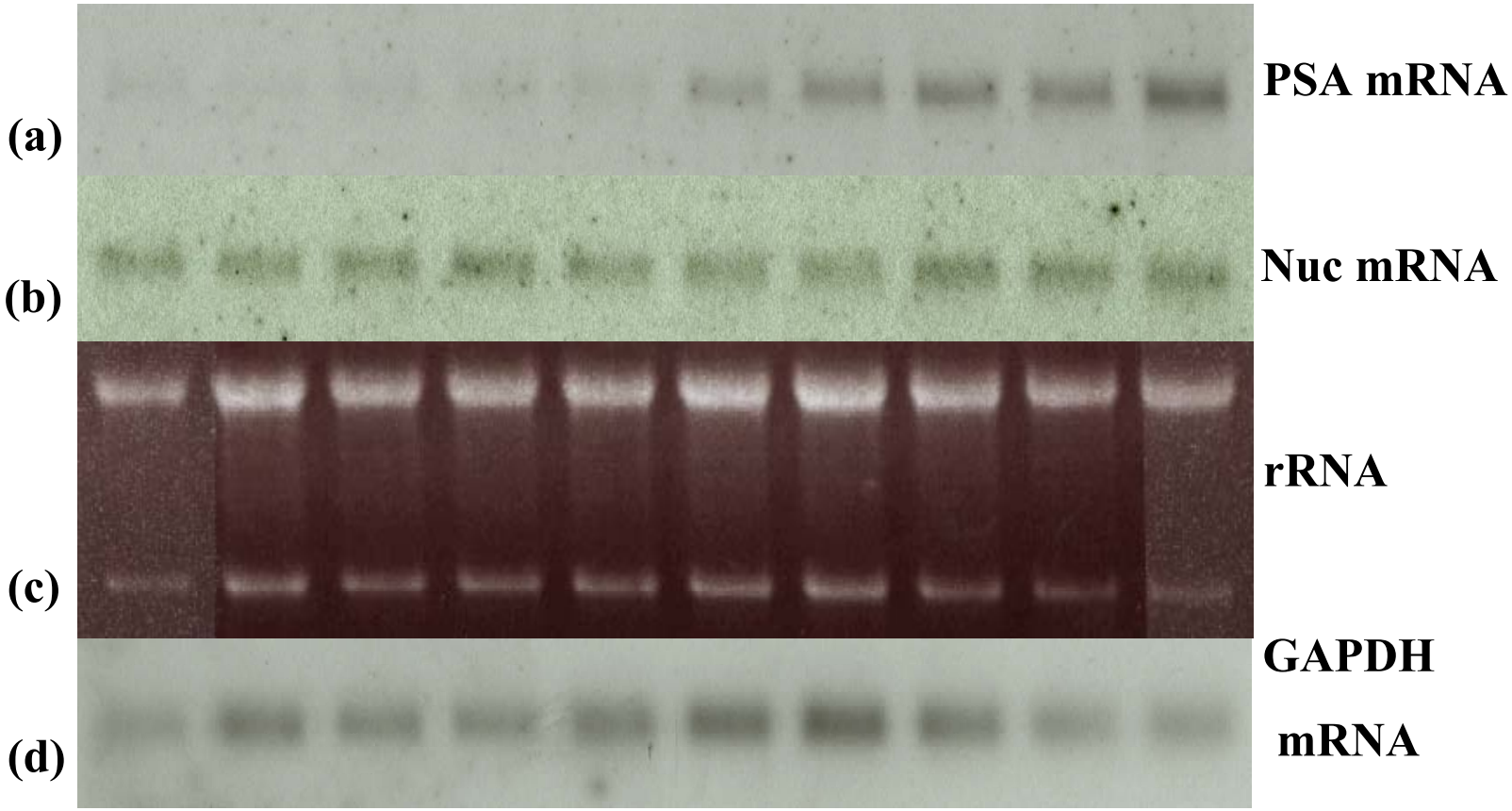

(a)
PSA mRNA
(b)
Nuc mRNA
rRNA
(c)
GAPDH
(d)
mRNA
0
0.5
1
2
4
8
16
24
32
48
Hrs AgR

Fig 2 The nucleolin gene is transcriptionally responsive to serum factors. A Crl cells were passaged and after two days in culture, were serum-starved. The culture medium employed for serum starvation was that which we normally use for Crl, only without FCS. Before addition of this FCS-depleted medium, the cells were washed 2x with PBS to remove any trace of the FCS that was in the medium used for passaging. The cells were left in incubation for 4 days. On the fifth day, the cells were serum-rescued through the use of the same type of medium to which 10% FCS was added. During the immediate hours of serum rescue, cultures were removed at the specified time points (one culture/time point) and lysed for RNA extraction. A Northern was then performed, assaying for Nucleolin mRNA. 1st row, Nucleolin mRNA 2nd row, 28S and 18S rRNA as our loading control 3rd row, GAPDH mRNA as a visual loading control B Data derived through the same serum-starvation/serum-rescue experiment using Npt and Cpt cells. The period of serum deprivation lasted 3 days. 1st row, Nucleolin mRNA 2nd row, rRNA as the loading control. 'SR' denotes serum rescue.

Fig 2

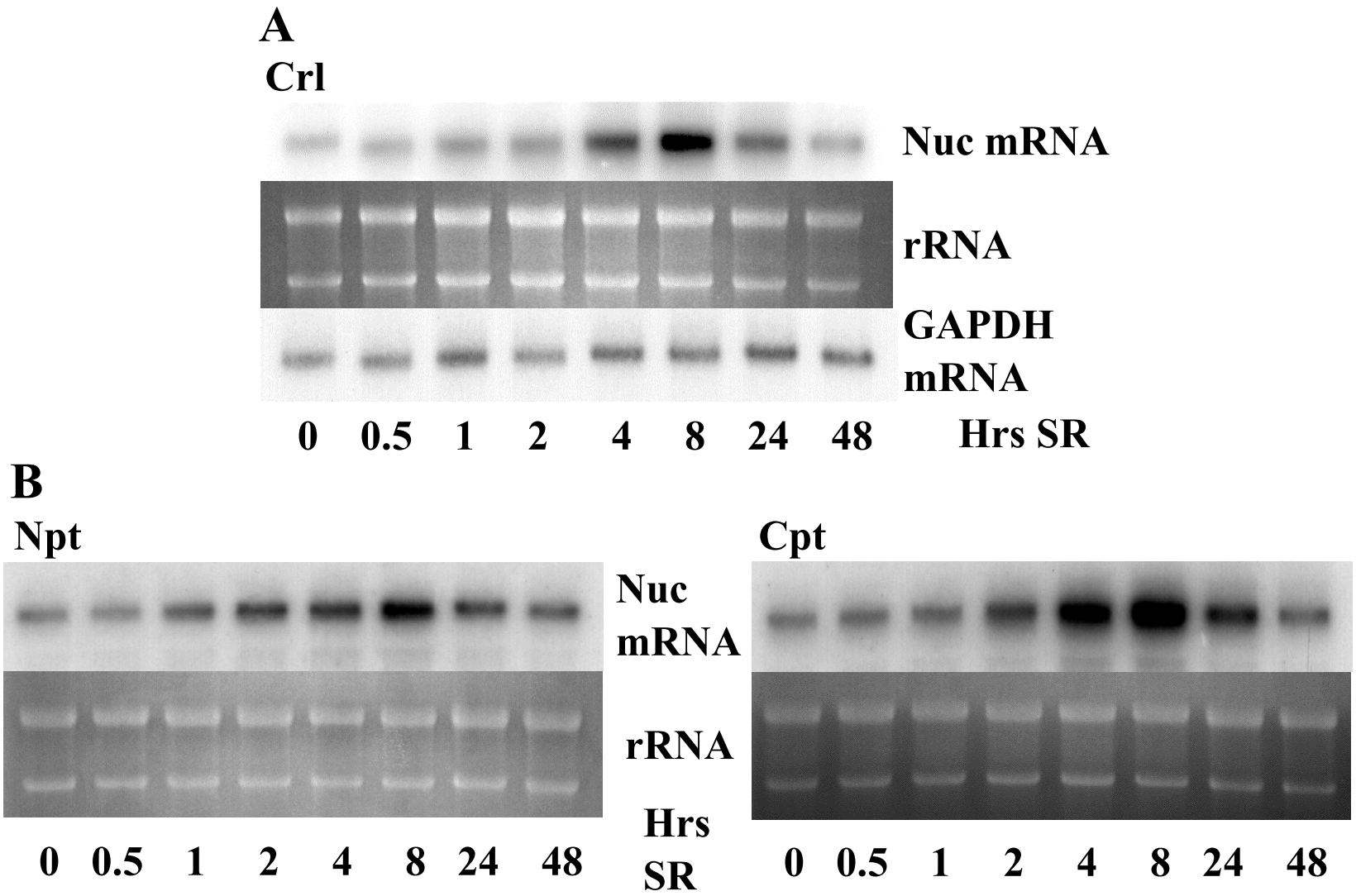

A
Crl
Nuc mRNA
rRNA
GAPDH mRNA
0 0.5 1 2 4 8 24 48 Hrs SR
B
Npt
Cpt
Nuc mRNA
rRNA
Hrs SR
0 0.5 1 2 4 8 24 48
0 0.5 1 2 4 8 24 48

Fig 3 The Nucleolin mRNA level remains constant in LnCp cells even after a period of serum starvation and during serum rescue. LnCp cells were passaged and after two days in culture, were serum-starved. The culture medium employed for serum starvation was RPMI with 0% serum. After 2 PBS washes, serum starvation was begun upon addition of the FCS-depleted RPMI, and lasted for 7 days. On the eighth day, the cells were serum-rescued through the use of RPMI to which 10% FCS was added. During the immediate hours of serum rescue, cultures were removed at the specified time points (one culture/time point) and lysed for RNA extraction. A Northern was then performed, assaying for Nucleolin mRNA. (a) Nucleolin mRNA (b) 28S and 18S rRNA as our loading control.

Fig 3

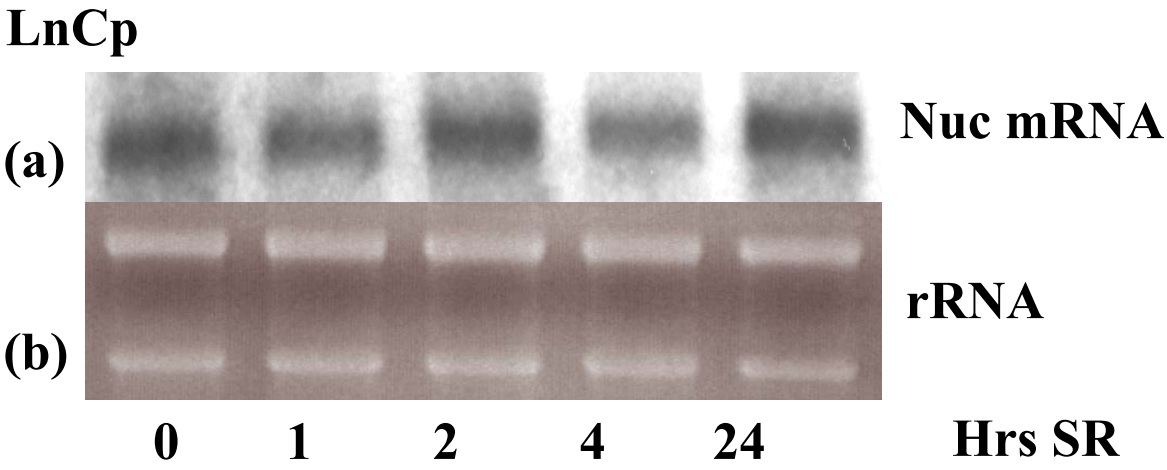
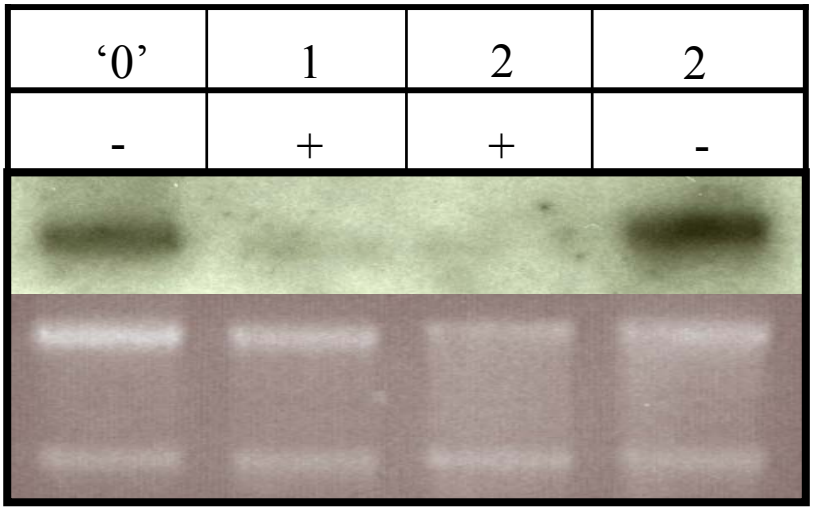


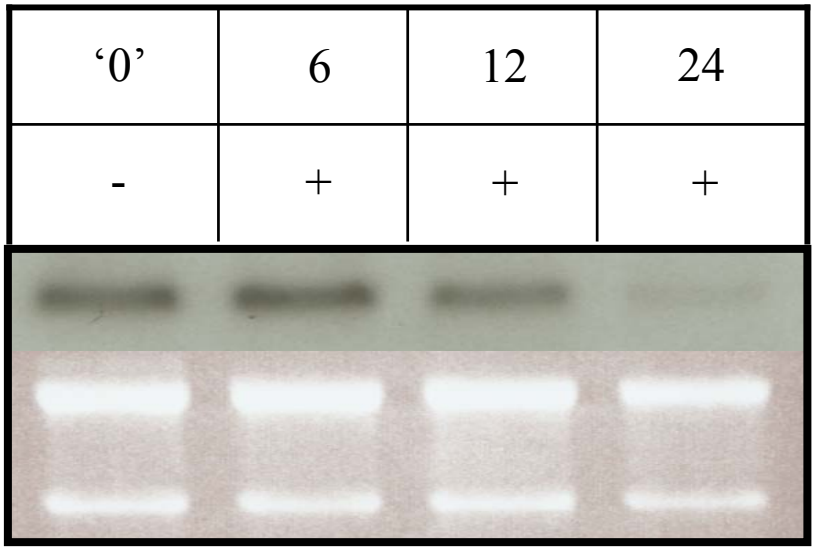
Fig 4 The constancy in the level of Nucleolin mRNA is not due to aberrant RNA stabilization. (a) Asynchronously-growing Crl cells were treated with actinomycin D for the periods of time indicated. Actinomycin D (ActD) was used at a concentration of 1 □g/ml. After RNA extraction and preparation, a Northern was performed, assaying for the presence of Nucleolin mRNA. Lane 1 control-cell population to indicate the level of Nucleolin mRNA after passaging of cells; lane 2 cells that were treated with ActD for 1 day; lane 3 cells that were treated for 2 days; lane 4 another control culture that had been left in incubation for the 2-day experiment, to show that Nucleolin-mRNA level was not affected by culture conditions or the incubator. (b) Asynchronously-growing LnCp cells were treated with ActD at a concentration of 1 μg/ml, for the indicated periods of time. Lane 1 represents a control experiment to show Nucleolin-mRNA level in cells after passaging, and lane 5 represents another control experiment to show that the mRNA level was not affected by culture conditions or the incubator during the duration of the experiment. (c) LnCp cells were simultaneously serum-starved and treated with ActD. Lane 1 represents a control experiment to show Nucleolin-mRNA level in cells after passaging, and lane 4 represents another control experiment to show that the mRNA level was not affected by culture conditions or the incubator during the duration of the experiment; lane 2 represents cells that had been serum-starved and were then treated with drug vehicle; lane 3 represents cells that had been serum-starved and then were ActD-treated.

Fig 4

(a) Crl



(b) LnCp



(c) LnCp

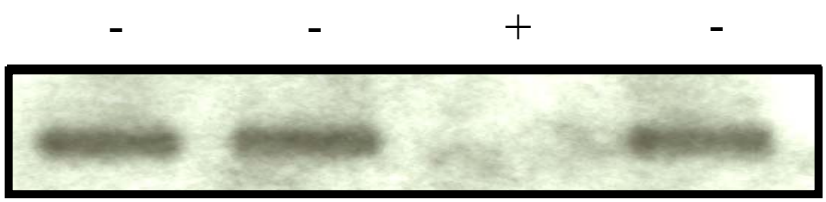


Fig 5 The level of Myc protein is constant in LnCp cells. A Crl and B LnCp cells were serum-starved, and then during serum rescue, cells were collected at the indicated time points for protein extraction (1 cell culture/time point). Protein was extracted as described (Materials&Methods). The samples were run through a 12% SDS gel and then transferred to a nitrocellulose blot, which was subsequently cut in half. One half was probed with an anti-Myc antibody (Ab), and other half, determined through the use of molecular markers to contain Actin, was probed with an anti-Actin Ab. The level of Actin was used as a loading control.

Fig 5

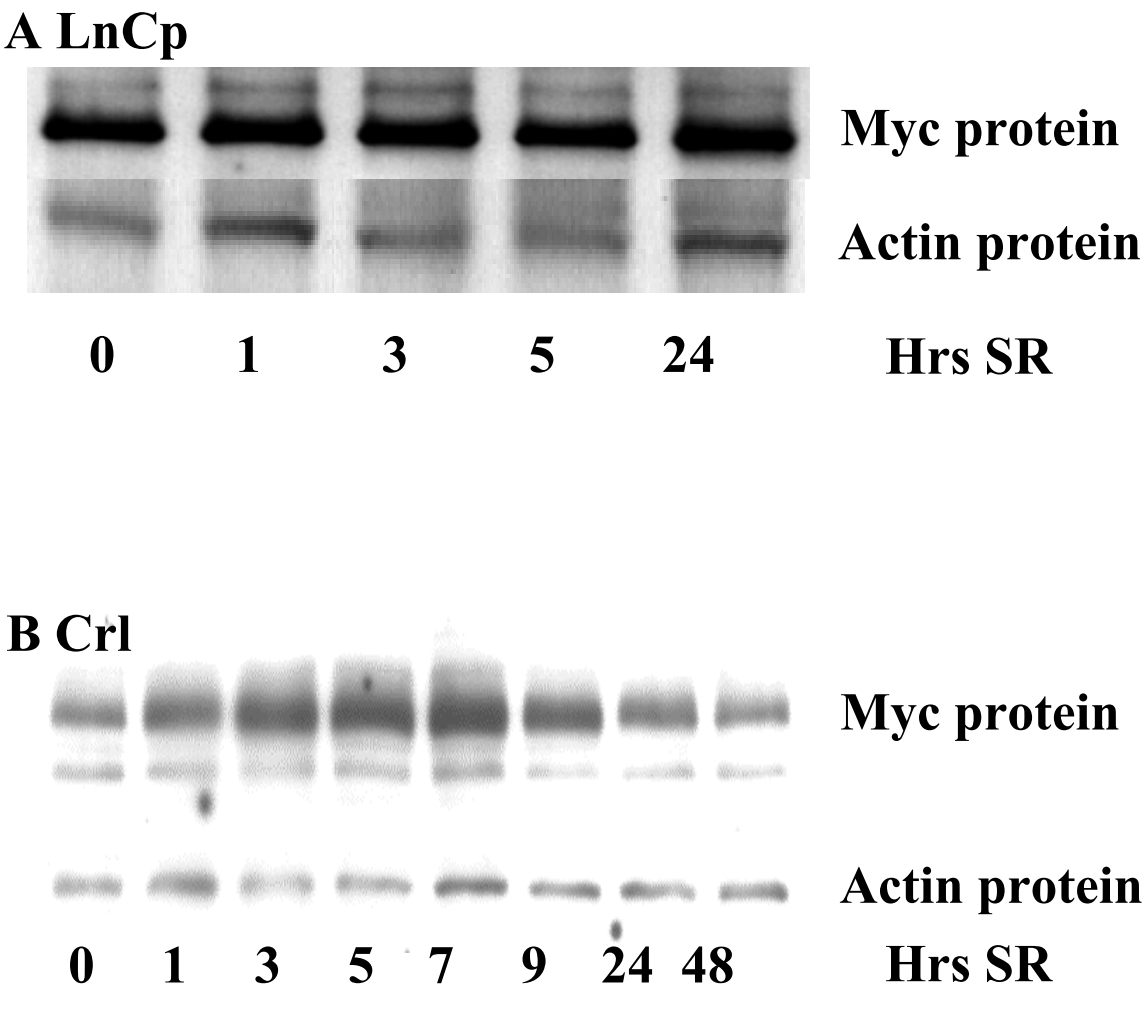

A LnCp
Myc protein
Actin protein
0
1
3
5
24
Hrs SR
B Crl
Myc protein
Actin protein
0
1
3
5
7
9
24
48
Hrs SR

Fig 6 Myc protein does not appear to be responsible for the constant Nucleolin mRNA level in LnCp cells. A PC3 cells were serum-starved (5 days), and then during serum rescue, cells were collected at the indicated time points for both RNA extraction and protein extraction (1 cell culture/RNA or protein extraction/time point). RNA was prepared through the Trizol method, and protein was extracted as described (see Materials&Methods for more detailed discussion of both protocols). Top half of figure, A Northern was performed to assay for the level of Nucleolin mRNA, using a probe specific to the amino-terminus of nucleolin cDNA. Bottom half, Western blotting was conducted and the level of Myc protein assayed for using a Myc-specific antibody (for more detailed description, see Materials&Methods). B Rat1a, a rodent fibroblast cell line and a subline, designated by us as Myc-/-, (for more detailed discussion of both cell lines, see text in Results section) were serum-starved and then serum rescued. As described before, during the serum-rescue period, RNA was extracted from culture at specific time points (1 cell culture/time point). A Northern was performed, assaying for expression of Nucleolin mRNA.

Fig 6A

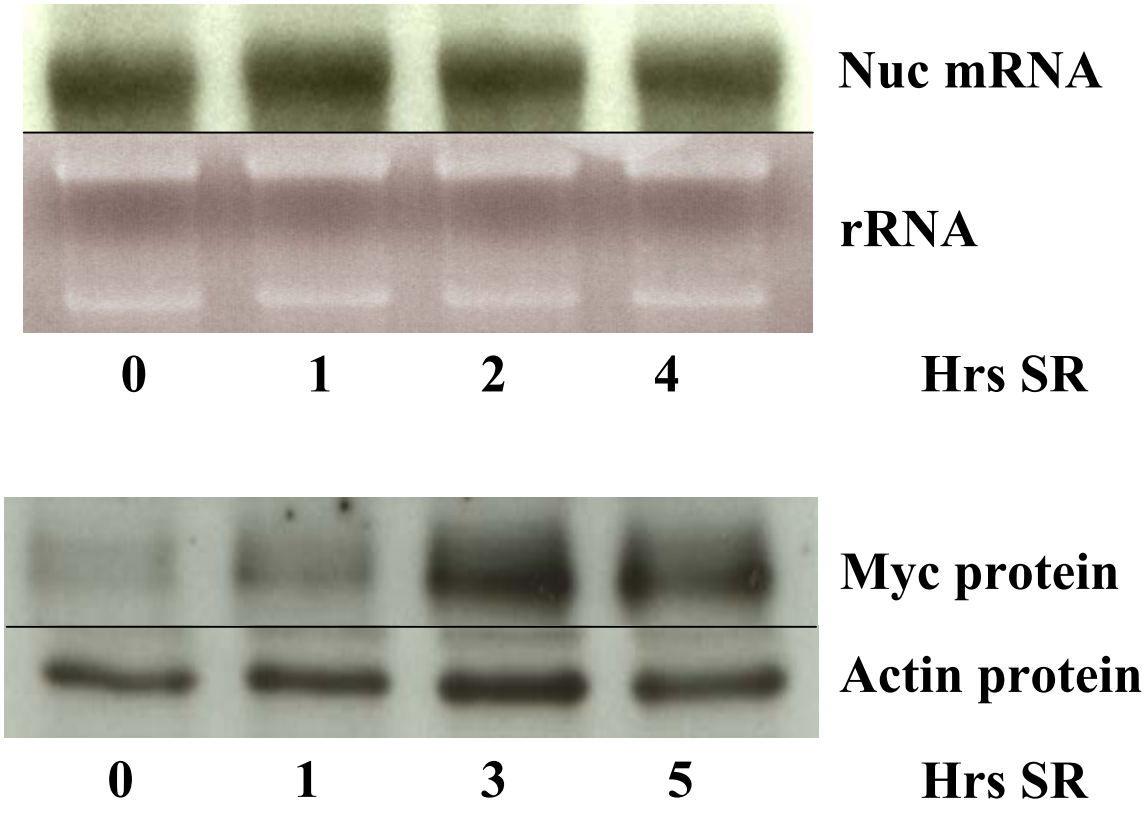

PC3
Nuc mRNA
rRNA
0
1
2
4
Hrs SR
Myc protein
Actin protein
0
1
3
5
Hrs SR

Fig 6B

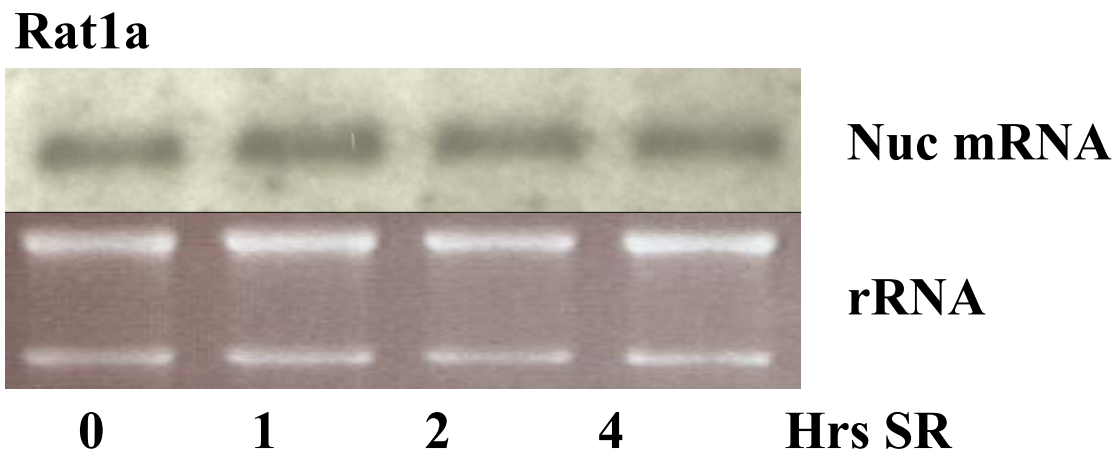
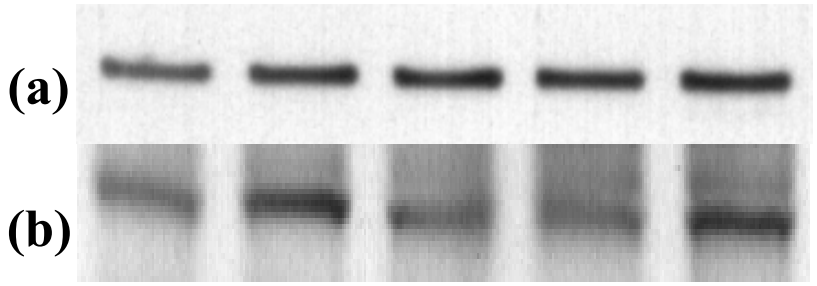


Fig 7 The level of Nucleolin protein is constant in LnCp cells as expected. LnCp cells were serum-starved, and then during serum rescue, cells were collected at the indicated time points for protein extraction (1 cell culture/time point). Protein was extracted as described (Materials&Methods). This figure presents a Western blot that was first shown in Fig 5. The half that had been probed with the anti-Myc Ab was stripped and then incubated with an anti-Nucleolin Ab. The Actin loading control is the same as that displayed in Fig 5.

Fig 7



RESULTS

The *nucleolin* gene is not transactivated by the androgen receptor that is bound by androgen in an androgen-dependent prostate metastatic cell line Tawfic *et al* (Tawfic et al 1994) employed castrated rodents to assay for the effect of androgen on the synthesis of Nucleolin. We used a culture-based system to test for and confirm this effect. Unlike normal prostate epithelial cells within the intact prostate organ, LnCp cells had not been surrounded by prostate stromal cells during their metastatic spread, before they were extracted from bone for placement in tissue culture. The cells had been directly exposed to serum. We reasoned, therefore, that this cell type has been acclimated to direct, unimpeded contact with androgen at its normal blood concentration (see Discussion). Tissue-culture medium with androgen may, in this regard, mimic the blood milieu to which LnCp cells have been exposed in their history.

After placing LnCp cells in culture, we deprived them of androgen by washing the cells with PBS to remove all traces of androgen and then replacing the medium normally used for this cell type with medium containing charcoal-stripped FCS (from which androgen had been removed). After allowing sufficient time for androgen-receptor (AR) activity to diminish, we 'rescued' the cells by providing them with the same medium to which, at this time, agonist was added. We used androgen at a concentration of 10^{-10} M, a dose that is established to stimulate androgen-dependent cell proliferation. Because we wished to detect direct transcription of the *nucleolin* gene by androgen receptor, we assayed for a change in Nucleolin mRNA synthesis, through a Northern, and attempted to detect this change, if it occurred, during the immediate hours following androgen stimulation. Our positive control was the expression of PSA mRNA, PSA being a documented target for AR. In contrast to PSA mRNA (Fig 1a), which clearly was no longer being synthesized by the end of the androgen-deprivation period, Nucleolin mRNA could be detected (Fig 1b). Also, its level remained the same through the period after agonist addition to the cells (Fig 1b). There was no rise in the level to indicate that the *nucleolin* gene was being transcribed by AR bound with agonist.

The *nucleolin* gene appears to be transcriptionally stimulated by serum in normal prostate cell lines and a cell line derived from a non-metastatic carcinoma It has been found by others that the *nucleolin* gene is transcriptionally silent when cells are growth-

inhibited, but becomes active when the same cells are stimulated to proliferate. Serum has been shown to have an inductive effect on the transcriptional state of *nucleolin*. Therefore, we reasoned that the constant presence of Nucleolin mRNA in our androgen-deprived LnCp cells was due to some serum factor(s), besides androgen, that activated a signaling pathway in the LnCp cells to maintain transcription of the *nucleolin* gene. Our next undertaking then was first to show that the gene was serum-responsive in cell lines derived from normal prostate tissue – Crl and Npt – and possibly in a cell line from a non-metastatic carcinoma – Cpt. After placing cells in culture, we washed the cells with PBS to remove all traces of FCS. We then bathed the cells in regular medium containing no serum (0%). We had determined earlier the time of serum starvation that was sufficient to inhibit cell proliferation (through FACS analysis of DNA content, data not shown). After leaving the cells deprived of serum for this time period, we serum-rescued the cells (culture medium with 10% FCS) and assayed for Nucleolin mRNA through a Northern. As can be seen from the RNA data, there is a clear rise in its level, reaching a peak at 8 hrs (Fig 2A & 2B) (A higher level may possibly be obtained by assaying for Nucleolin mRNA at later time points, and so we do not wish to state that the actual peak occurs at 8 hr.). Because we did not directly test for transcriptional activity -- through an experiment like a nuclear run-on assay -- we cannot definitively state that serum induced transcriptional initiation at the *nucleolin* locus in our cells. This is a necessary precautionary interpretation since in one study Westmark and Malter found that mitogen stimulation of PBMCs led to the stabilization of Nucleolin mRNA rather than its synthesis; nevertheless, the rise in mRNA level had the appearance of stimulated transcription of the *nucleolin* gene (Westmark and Malter, 2001).

The presence of Nucleolin mRNA appears to be serum-independent in the LnCp cells We performed the same serum-starvation/serum-rescue (S/R) experiment with LnCp cells. To our surprise, Nucleolin mRNA remained at a constant level, even after serum starvation (Fig 3). Hence, we were forced to reinterpret our earlier finding of this same constancy after androgen deprivation. As discussed above, we assumed that the constant level was due to constitutive transcription of the *nucleolin* gene through a serum factor other than androgen (the most downstream element in an intracellular signaling pathway).

However, the experiment under focus here indicates that the presence of Nucleolin mRNA, if not its synthesis, is independent of all serum factors.

The stability of Nucleolin mRNA is the same between LnCp cells and Crl cells, and the mRNA is not aberrantly stabilized in LnCp cells To get a rough indication of Nucleolin-mRNA stability, we assayed for the mRNA after a relatively long period of a day, and consecutive days after, following an application of actinomycin D, a drug that inhibits RNA synthesis, to Crl cells. Our assumption was that a result obtained using Crl cells would be reflective of the normal Nucleolin mRNA state since the serum-sensitivity of its level in this cell type also conformed to past data derived by others. The Northern data indicate that the half-life may be 12 hrs or even less (Fig 4a). The Crl data would be our point of comparison, and, therefore, we next attempted to detect the stability in LnCp cells. We confined drug treatment to a day, since the previous result showed mRNA degradation by one day after such treatment. We also assayed for Nucleolin RNA at earlier time points within the day to obtain a more precise determination of its half-life. As expected, the half-life appears to be approximately 12 hr (Fig 4b).

The experiments outlined above were performed on asynchronously-growing cells. However, the result from the S/R experiment with the LnCp cells showed the presence of Nucleolin mRNA after serum starvation. The possibility remained that the mRNA is aberrantly stabilized in this particular condition. Therefore, we serum-starved LnCp cells and on the final day of the deprivation period, simultaneously starved and treated the cells with actinomycin D. The treatment period was for one day. Our reasoning was that if there were aberrant stabilization, Nucleolin mRNA should be detectable after treatment. Our data shows that the mRNA stability remained the same in the absence of serum (Fig 4c).

Myc protein is constant in level in LnCp cells It has been independently demonstrated by five groups that overexpressed Myc protein can transactivate the *nucleolin* gene. In the S/R experiment involving LnCp cells, we also extracted protein from the serum-rescued cells, with which we then performed an immunoblot assay. In contrast to results derived by other research groups, our data show an unexpected expression profile of Myc (Fig 5A). The protein showed no drop in its level after serum starvation and there appears to be no synthesis of the protein following serum rescue. For comparison, we

include data derived through same S/R experiment using Crl cells (Fig 5B). The expression profile of Myc in this case is the expected one. With regard to Myc from LnCp cells, its constant level might indicate its constant activity as a transcriptional factor. Therefore, we reasoned that the constancy in the Nucleolin-mRNA level might be due to Myc constancy. If endogenous Myc can transactivate the *nucleolin* gene in the same way that ectopically-expressed Myc can, it might be possible that Myc protein that we observed in serum-starved LnCp cells is maintaining transcriptional activity at the *nucleolin* gene under this particular condition.

Endogenous Myc protein does not transactivate the *nucleolin* gene We used another prostate metastatic cell line known as PC3 for the S/R experiment. Our original purpose was to determine whether or not the same constancy in Nucleolin mRNA might be observed in this particular cell line following a period without serum. If we did derive the same result using this cell line, we reasoned that we might more comfortably begin to associate this phenomenon with the metastatic phenotype exhibited by both LnCp cells and PC3 cells. In fact, the mRNA level did stay constant, even after serum starvation and during serum rescue (Fig 6A *top half*). However, to our surprise the expression profile of Myc protein (Fig 6A *bottom half*) was similar to that of Myc from Crl cells in the same S/R experiment. With regard to the question of whether or not endogenous Myc protein does in fact transcriptionally activate the *nucleolin* gene, the S/R data from PC3 cells is illuminating. While Myc protein dropped to a relatively low level, Nucleolin mRNA stayed at the demonstrated level, indicating that the presence of the mRNA does not require Myc protein. During serum rescue, as the Myc level rose, there was no rise in Nucleolin mRNA, as would be expected if the gene were being transcriptionally activated by Myc. If Myc were acting as a *nucleolin* transcription factor, a temporal correlation should be found between the expression profiles of Myc protein and of Nucleolin mRNA. A peak in the level of Myc should be followed immediately by a similar peak in the level of Nucleolin mRNA. We observed no such correlation, and from this data, we tentatively concluded that the *nucleolin* gene is not a target of Myc in PC3 cells.

To confirm this finding, we subjected a pair of rodent fibroblast cell lines to the S/R experiment. The Myc-/- cell line (our designation, to simplify the discussion) was derived from the parental Rat1a cell line. The endogenous *myc* gene was deleted in

cultured Rat1a cells through homologous recombination (Mateyak et al 1997). There is no evidence that Myc family members, L-Myc and N-Myc, rescue the phenotype that results from this gene deletion. This particular cell line has been employed in studies to determine the validity of other genes as Myc targets. Our data show that the *nucleolin* gene could appear independently of Myc during serum rescue (Fig 6B). It does appear that the kinetics of mRNA appearance is affected by the absence of Myc (compare lanes 1 and 2 of the bottom-half Northern blot), suggesting that in fact Myc may facilitate the transcription of *nucleolin* (or the increased stability of its mRNA). However, the mRNA was able to reach a steady-state level fairly quickly and was able to remain at this level despite the absence of Myc. Therefore, we conclude that Myc is unnecessary for the expression of Nucleolin. If, in fact, the constancy of Nucleolin mRNA in our LnCp cells is due, at least partly, to Myc that remains even after serum starvation, then this instance of Myc 'localization' to the *nucleolin* locus must be specific to LnCp cells. Because we had no clean and reliable way to inactivate Myc in our LnCp cells, we could not definitely address this possibility.

Nucleolin protein is constant in level in LnCp cells Because the level of Nucleolin mRNA is constant, we predicted that its protein would be constant in its level. Using the same blot from the S/R experiment involving the LnCp cells discussed above (Fig 3), we stripped it of the Myc-specific antibody and then applied a Nucleolin-specific antibody to it, assaying for its expression profile. As expected the protein level was in fact constant (Fig 7).

DISCUSSION

Our focus in this paper has been to understand an aberration in the expression pattern of Nucleolin mRNA that we have detected in a prostate metastatic cell line. We demonstrated that cell lines derived from healthy prostate tissue and a cell line from a prostate non-metastatic carcinoma show the characteristic expression profile of Nucleolin mRNA that has been documented and is to be expected from cells in culture after a particular treatment. We make no claim, however, that what we were able to observe in LnCp cells regarding Nucleolin mRNA is a phenomenon that is specific to prostate metastasis. LnCp cells are an old cell line, and some of the characteristics of Nucleolin mRNA in this cell type might have resulted from a combination of cell-culture adaptation and the particular intracellular signaling that this strain (keeping in mind the possibility that different labs working with LnCp cells may possess different subtypes of LnCp cells) of LnCp cells employs for its proliferation and survival in culture.

Our original intent was to confirm the finding that the *nucleolin* gene is regulated in prostate epithelial cells in a specific way, being directly activated by the androgen receptor (AR) when bound with dihydrotestosterone. We wanted to show the feasibility of a cell-culture system not only for this purpose but also to elaborate upon it by determining what other factors besides Nucleolin are similarly affected by androgen. We wished to understand the biological consequence of this particular gene-expression profile. To reiterate what was discussed earlier, our system differed from that used by Tawfic *et al*, in important ways. Prostate epithelial cells in our culture system were not surrounded by prostate-specific stroma. Cells of prostate stroma express AR and respond to circulatory androgen (Cunha et al 1987; Cunha 1996; Hayward et al 1997). Although prostate epithelial cells respond to androgen, they appear to do so by secreting seminal proteins. The evidence from work concerning prostate development is that these epithelial cells grow and proliferate as an indirect response to androgen. Surrounding fibroblasts are stimulated by androgen to secrete growth factors that go on to induce, through a paracrine mode, a proliferative response by the epithelial cells. In fact, higher doses of androgen are known to inhibit proliferation of these cells. It is possible that the lower doses required are achieved through a titration effect that the prostate fibroblasts have by virtue of their position in prostate-tissue architecture – in closer proximity to

blood vessels relative to the epithelial cells, and surrounding the epithelial cells – thereby being exposed first to circulatory androgen. Unbound androgens that go past the stromal layer are at a relatively lower concentration, and it may be this amount that prostate epithelial cells require to activate the genes encoding secretory factors. In our system the androgen agonist was added directly to the cell-culture medium in which the LnCp cells were bathed. Our justification for this approach is that LnCp cells are of a metastasis that require androgen for their growth and survival. As they metastasized, the cells had been directly exposed to blood(serum)-borne factors while moving to bone from which they were extracted for tissue-culture seeding. In our system we used transcriptional upregulation of the gene encoding PSA to assay for transcriptional activity on the part of AR. It is possible that transcription of the *nucleolin* gene is androgen-sensitive, but only indirectly, and that it is through a growth factor(s) normally secreted by prostate stromal cells in response to androgen that Nucleolin mRNA synthesis occurs. In the absence of such a growth factor(s) in the cell-culture medium that we used for the LnCp cells, the *nucleolin* gene would be transcriptionally silent, even after addition of the androgen agonist.

As mentioned above, LnCp cells require androgen for their proliferation and appear to respond directly to androgen to grow and proliferate. If the hypothesis that we discuss is correct, that prostate epithelial cells normally respond indirectly to androgen to proliferate, it is interesting that LnCp cells have not co-opted the use of AR to directly stimulate the transcription of a gene like that encoding Nucleolin. Nucleolin facilitates ribosome biogenesis, and so its promotion of cell growth might be of selective advantage to an aggressively-growing cell type like LnCp cells. Therefore, use of AR to directly activate the *nucleolin* gene might provide this advantage to a cell in a heterogeneous collection of cells within a prostate carcinoma, assuming the re-distribution of AR from its normal targets to targets that are outside its normal target-gene repertoire is genetically stable and therefore heritable. It could be that this, in fact, has occurred in LnCp cells and may have contributed to their ability to metastasize. Constitutive transcriptional activation of the *nucleolin* gene that is mediated by AR may have been unnecessary because the gene was already activated through another pathway, possibly involving a transcriptional co-activator whose activity is normally tightly regulated, but, in cells of a

metastasis, is constitutively on.

Previous work has shown that the *nucleolin* gene is sensitive to serum factors. During cell quiescence Nucleolin-mRNA synthesis is downregulated, and following the addition of serum, transcription of the *nucleolin* gene appears to commence. We confirmed this result through an examination of our normal prostate cell lines. Therefore, we wanted to determine what besides androgen in serum could be responsible for this effect on the level of Nucleolin mRNA in our androgen-deprived LnCp cells. Our examination of LnCp cells, using the same serum-deprivation/serum-rescue protocol, yielded a surprising result: as with androgen-deprived LnCp cells, serum-starved LnCp cells showed no drop in the level of Nucleolin mRNA; and serum-rescued LnCp cells did not appear to synthesize more Nucleolin mRNA. One possible explanation for the presence of mRNA even after serum starvation is that the *nucleolin* gene was amplified. If the extra copy were constitutively on, being 'outside' the regulatory mechanism used for the endogenous gene, possibly by virtue of its location in the genome, the mRNA would always be synthesized. It is not always the case that extra copies of a gene within an amplicon are expressed; the gene may be copied, but not its promoter region. When a gene along with its regulatory region is duplicated, its placement outside the locus of the endogenous gene – as a result of the recombination mechanism that leads to duplication – may allow it regulatory independence and may even result in its being under the control of another enhancer element. We did not investigate this scenario because we were more interested in determining whether or not the constancy of Nucleolin mRNA was due to an enhanced rate of transcriptional initiation at its endogenous promoter/enhancer rather than to an altogether different mode of transcriptional regulation. Also, we wanted to determine whether or not the constancy might be directly attributed to a change in the mRNA itself.

Another possibility we considered was that the Nucleolin mRNA was aberrantly stabilized in LnCp cells. Stability of a factor, whether an mRNA or protein, may be contingent on the extracellular environment with which the cell interacts (see Wilusz et al 2001 for review). It is possible that the mRNA for a protein like Nucleolin may be stabilized under growth-limiting conditions so that the protein may always be synthesized even in such conditions. The maintenance of its presence might ensure its constant

activity. Although ribosomes are not required to the same extent during cell quiescence, a ribosome-assembly factor like Nucleolin may be kept at a constant level so that, upon re-stimulation of the cells to grow and proliferate, ribosome assembly may immediately occur to meet the quick demand for protein by cells. If there were no transcription of the *nucleolin* gene in LnCp cells even after 7 days without serum, and mRNA stability accounted for its constant presence, then that stability must be either environment-induced or some sort of aberration. Enhanced stability in response to the environment may itself be an aberration that was selected for. We found that the half-life of Nucleolin mRNA is 12 hrs, and so its normal stability cannot account for its presence after a 7-day serum-deprivation period, assuming no ongoing transcription of the gene. We found no indication of aberrant stabilization. During serum starvation of LnCp cells, the mRNA displayed the same stability it possesses in asynchronously-growing cells. Also, the strain of LnCp cells we worked with could not have been selected for because of its ability to stabilize the mRNA for a factor like Nucleolin. This would imply that during its history as a cell line, it had been maintained under minimal serum levels and a subline eventually emerged that could survive under such pressure because of its ability to maintain a constant level of growth-promoting factors such as Nucleolin. We always maintained a stock of LnCp cells, and cells that were used for any experiment, including the serum-starvation experiment, were discarded if not harvested for RNA and protein extraction (and for other purposes).

Considering the lowering of the Nucleolin mRNA level under a similar condition exhibited by the normal prostate cells, the question then focused on the ability of LnCp cells to maintain transcriptional activity at this gene. Autocrine stimulation of this gene is one possibility. The autocrine mode may be satisfied through any number of molecular mechanisms. For instance, cells may secrete a factor(s) that may or may not be found in serum, and the cells may be able to respond to this factor through cell-surface receptors that it possesses. We did make an attempt to test this possibility by bathing serum-starved Crl cells (our normal prostate cell line) with conditioned FCS-depleted medium of LnCp cells. We found no upregulation in the level of Nucleolin mRNA as a result (*data not shown*). However, this particular experiment has the important caveat that the Crl cells may not possess the receptor(s) for the secreted factor(s) or may possess an

inadequate number of such a receptor for a vigorous response. Another autocrine mechanism may be constitutive ligand-independent activation of the signaling pathway that normally leads from an activated cell-surface receptor to the enhancer-binding factors and co-activators that initiate transcription of the *nucleolin* gene.

Another autocrine mode in the maintenance of transcriptional activity is the constitutive activation of the transcriptional factor that initiates transcription of the gene in question. We considered the possibility that Nucleolin-mRNA constancy was due to the constancy in the level of Myc protein in LnCp cells. In our normal prostate cell lines, there is a loose temporal correlation between the expression profiles of Myc protein and of Nucleolin mRNA. As Myc levels rise, Nucleolin mRNA levels also rise (Fig 5B). However, in the LnCp cells there is no downregulation of Myc protein after serum starvation. Given the documentation that Myc can transactivate the *nucleolin* gene, we considered the possibility that the presence of Nucleolin mRNA was due to its being transcriptionally on as a result of constitutive Myc binding to an E-box element within the *nucleolin* regulatory region. The first indication we had that this in fact may not be the case came from work with another prostate metastatic cell line called PC3. Like LnCp cells, this cell type showed no serum effect on the level of Nucleolin mRNA. The level stayed constant after serum starvation and during serum rescue. However, unlike LnCp cells but like the normal prostate cells, PC3 cells displayed the expected Myc expression profile; that is, the level dropped after serum starvation, but rose during serum rescue. The important points to note in the data are two: 1) the presence of Nucleolin mRNA despite the absence of Myc protein at the end of serum starvation – this indicates the ability of the cells to maintain Nucleolin mRNA independently of Myc protein; 2) the absence of upregulation in Nucleolin mRNA despite the rise in Myc levels – if Myc were to transactivate the *nucleolin* gene in the PC3 cells, the level of Nucleolin mRNA should rise following in close temporal order the rise in Myc protein. We then attempted to confirm this finding with our work on a pair of rodent fibroblast cell lines. The Myc-/- cells represent a subline of the parental Rat1a cells. There is no indication that family members L-Myc or N-Myc have rescued the *myc*-null phenotype of the Myc-/- cells. Also, the cells do display the expected effect of Myc absence: the cells proliferate at a slower rate with extended G1 and G2 phases. Both cell types were subjected to the

serum-starvation/serum-rescue protocol that we have employed throughout this project. Nucleolin mRNA is present in the Myc-/- cells, indicating a Myc-independent ability to synthesize Nucleolin mRNA.

One interesting point to note is the slow growth of the Myc-/- cells despite the presence of Nucleolin. If the role of Nucleolin in promoting cell growth is restricted to its activity in ribosome biogenesis, the slow-growth phenotype of the Myc-/- cells indicates that ribosome assembly is not rate-limiting for accumulation of protein on a global scale. The more direct effect of Myc may therefore be in translation initiation, either through transactivation of genes encoding initiation factors or through a post-transcriptional mechanism involving participants in translation initiation.

We have no explanation as to why the level of Nucleolin mRNA does not rise during serum rescue in LnCp cells. It is possible that with the level already being relatively high, the level may be at a saturation point, past which cells prevent further upregulation through a stringent regulatory mechanism. Nucleolin has been documented to act as a transcriptional repressor (Yang et al 1994), and it is possible that it may be part of an autoregulatory mechanism, inhibiting transcription of its own gene by binding a specific regulatory region when the amount of Nucleolin protein exceeds the permitted level. We are currently investigating this possibility.

The main question remains concerning the reason for this constancy in level of both Nucleolin mRNA and the protein in LnCp cells. mRNA presence is likely to ensure the constant presence of the protein, unless the mRNA performs a non-coding function. There is no evidence to indicate that this might be so. As discussed above, it is unlikely that these cells have adapted to minimal serum levels in culture. More likely is that, as one step in the acquisition of the metastatic phenotype, a subset of cells acquired the ability to grow independently of exogenous growth factors by synthesizing and secreting the necessary growth factors and their receptors. Such cells that were capable of this autocrine-mode of growth stimulation may have been at a selective advantage. The continuous activation of intracellular signaling pathways ensured the presence of Nucleolin and other factors that facilitate growth and proliferation. We have found that LnCp cells stop dividing in the complete absence of serum. So serum is still a necessity, probably in furnishing survival factors. However, the constant presence of growth-

promoting factors like Nucleolin may allow for LnCp cells to proliferate at a relatively faster rate and to grow under sub-optimal conditions. The fact that their serum-deprivation period could be extended to seven days rather than the four days required to halt Crl growth is consistent with this idea. Again, we wish to reiterate the point made earlier that the constant Nucleolin level may in fact be a cell-culture adaptation that is peculiar to prostate cells. As cells do nothing else but divide in culture, any such adaptation would likely be that which promotes cell growth and proliferation, and so such an adaptation is not mutually exclusive with *in vivo* cancer-associated adaptations. However, the pressure for such an adaptation in the body as the cancer grows and spreads may not be as great. Therefore, a constant Nucleolin level may not be a metastasis-specific phenomenon.

The peculiarity we have found concerning the expression profile of Nucleolin mRNA and the protein is consistent with its ability to promote cell growth.

PAPER 2

ABSTRACT

Nucleolin is a nucleolar phosphoprotein that directly participates in all phases of ribosome biogenesis, including rRNA synthesis and its modification and the interaction between pre-rRNA and ribosomal proteins. The expression of Nucleolin mRNA and protein is an unexplored topic of research. We were drawn to the question when we found a disparity between the expression profile of the mRNA and that of the protein: in all our cell lines that were serum-starved and then serum-rescued, the protein level remained constant. In some cell lines, this constancy was maintained despite the lowering of mRNA level after serum starvation and the rise in its level during serum rescue. It appears that Nucleolin protein is unusually stable and that its stability is unaffected even in the face of growth-factor cessation. Translation of the protein does not appear to be ongoing during growth quiescence and therefore is probably not stimulated through factors besides growth factors, like nutrients that contribute to the make-up of serum-free culture medium. Hence, even in the absence of continuing synthesis of Nucleolin protein, the steady-state level of the protein can be maintained for a certain length of period during growth quiescence through its high stability. Also, the level of the protein is held constant even in the presence of its mRNA and this constancy is probably due to a regulatory mechanism – possibly autoregulation – in which the cell senses an adequate supply of Nucleolin protein and prevents further rise in its level, through any one of a number of mechanisms, like concomitant protein degradation and synthesis or translational repression. Ramifications of what appears to be a requirement to maintain a constant Nucleolin protein level are considered.

INTRODUCTION

Nucleolin is the most abundant protein of the nucleolus. A non-ribosomal component, it nevertheless contributes to ribosome activity by facilitating the assembly of the ribosome. Knockouts of yeast orthologues (the genes encoding Nsr1p in *S cerevisiae* and Gar2p in *S pombe*) reveal a deficit in 18S rRNA production and therefore 40S assembly (Girard et al 1992; Gulli et al 1995; Kondo and Inouye 1992). There is no equivalent genetic reagent in a mammalian system: no mammalian cell line exists with the endogenous *nucleolin* gene deleted, and no *nucleolin* gene-knockout mouse has been constructed to date. Therefore, it has not been possible to determine exactly the particular effect that the absence of mammalian Nucleolin may have specifically on ribosome biogenesis. It should be noted, however, that hamster Nucleolin was unable to complement a *nsr*-knockout *S cerevisiae* strain (Xue et al 1993). One piece of evidence suggesting a role in ribosome biogenesis for Nucleolin is its localization to the nucleolus, particularly the granular component (Lischwe et al., 1981; Escande et al., 1985) of this intranuclear organelle where processing and modification of the rRNA primary transcript are known to occur. Indeed, careful biochemical work with Nucleolin reveals an activity of modifying the rRNA primary transcript (Ginisty et al 1998). Also, the *nucleolin* gene contains introns with coding regions for two snoRNAs belonging to the two major families of snoRNAs, each effecting a particular modification of a particular rRNA nucleotide (Nicoloso et al., 1994; Bachellerie et al., 1995; Bachellerie and Cavaillé, 1998; Smith and Steitz, 1997). The Nucleolin protein is able to interact with both ribosomal proteins and rRNA (Serin et al 1996; Ghisolfi-Nieto et al., 1996; Bouvet et al 1998; Sicard et al 1998; Serin et al 1997; Allain et al 2000; Ginisty et al 2001). Nucleolin may be able to participate in these interactions simultaneously and in this way bring ribosomal proteins and rRNA in close enough proximity for them to interact and thereby form the rudimentary ribosomal subunit. This is suggested by the modular structure of Nucleolin, but has not been confirmed either in a biochemical system or *in vitro* in cultured cells.

Presently, there is no comprehensive work dealing with the synthesis of Nucleolin protein. Much work has been devoted to post-translational modifications of the protein, mainly phosphorylation that is mediated by casein kinase II in response to stimulation of cell proliferation (Belenguer et al 1989; Bouche et al 1994; Bonnet et al 1996). Also,

there is evidence to hint at a self-cleaving activity that Nucleolin possesses and may employ as cells adjust to quiescence (Chen et al 1991). A survey of studies dealing with Nucleolin activity leads to the conclusion that it is multifunctional, participating in numerous activities besides ribosome biogenesis. These activities necessitate the localization of Nucleolin outside the nucleolus, including the cell surface (Kleinman et al 1991; Hovanessian et al 2000). How cells adjust the translational rate of Nucleolin mRNA and the steady-state level of the protein in response to the demands of these different activities is also another neglected area in the Nucleolin field.

While working to understand the possible role that Nucleolin may have in the cell cycle, we came across an unexpected result showing a discrepancy between the expression profile of the Nucleolin mRNA and that of the protein. It has already been demonstrated by other groups that the *nucleolin* gene is transcriptionally sensitive to serum factors that activate a signaling pathway(s) in cells that leads to transactivation of the gene. We found in most of our prostate cell lines that this indeed was the case. However, to our surprise the protein level was constant at all times. During and even after a period of serum starvation, the protein level did not drop as expected. And at the immediate hours of the serum-rescue period, when the Nucleolin mRNA level reached a peak, there was no rise in the protein level, temporally following the rise in the mRNA to indicate active translation. We investigate in this paper the possible reasons for this constancy in the protein level, and under consideration were the following possibilities: 1, to accommodate the drop in Nucleolin mRNA synthesis during serum starvation, the cell somehow selectively enhances the translational rate of the protein, leaving no change in the steady-state level of the protein that is exhibited by asynchronously-growing cells; 2, the stability of the protein may be such that no drop in the steady-state protein level can be detected through a conventional Western, even with translational shut-off during the serum-deprivation period; 3, there is sustained protein synthesis that is stimulated by nutrients in the cell-culture medium rather than serum factors, and the Target of Rapamycin (TOR) factor may mediate the pathway activated by nutrients like amino acids that leads to the translational apparatus on the Nucleolin mRNA; 4, the apparent absence of translational upregulation of Nucleolin during serum rescue when the mRNA levels steadily rise is due to protein degradation that occurs concomitant with protein

synthesis, resulting in the same steady-state level; 5, there is indeed no translation of the protein during serum rescue, and this is due to Nucleolin self-regulation, in which the protein binds its own mRNA to repress translation. The last point indicates an excess of the protein. Like other RNA-binding proteins, Nucleolin may be subject to stringent regulation so that its level does not rise above the needed level. This point will be further discussed at the end of this paper.

MATERIALS & METHODS

Cells and reagents

The following cell line of the two prostate cell lines that were employed in this study was kindly provided by Dr. Shiv Srivastava of USUHS: LnCp. We acquired the other cell line, the Crl cell line, from the ATCC (CRL-221). Ingredients for cell-culture media are the following: 1 for LnCp cells, RPMI-1640 (I/LT) with 10% FCS (I/LT) and 1% Pen/Strep (I/LT); 2 for Crl cells, Defined Keratinocyte SFM (I/LT) with 10% FCS and 1% Pen/Strep.

The *myc* probe consisted of the entirety of the cDNA from the *myc* gene and was supplied to us by Dr. Michael Keuhl of the NCI. The cDNA had been subcloned into an expression cassette, and we cleaved this vector with EcoR1 to release the entire cDNA. The cDNA was then gel-purified following the Wizard DNA purification system (Promega). For our *nucleolin* probe we used an expression vector into which the *nucleolin* genomic DNA had already been subcloned. Using the PCR (Perkin Elmer) technique, we amplified the amino terminus. We then gel-purified this fragment using a similar Wizard kit designed for the purification of PCR fragments.

For Western blotting we employed the following antibodies (Ab): for Nucleolin, one Ab was purchased from Santa Cruz, and the other from MBL (Medical and Biological Laboratories, Co., LTD.); for Myc, the Ab was purchased from Santa Cruz; for Actin, the Ab was purchased from Santa Cruz; for Tubulin, the Ab was purchased from Santa Cruz. The secondary Ab was purchased from Amersham Life Science.

Cycloheximide and rapamycin were purchased from Sigma.

Assay for mRNA

RNA was extracted from cells and purified using Trizol (I/LT). Briefly, for a 100-mm culture dish, 1 ml of Trizol was added straight to cells after culture medium was removed and discarded. After lysing of cells was complete, the 1 ml was transferred to an eppendorf tube. The Trizol protocol that was provided with product was then followed. Briefly, the protocol involves lysing of cells through the Trizol reagent, followed by chloroform-based extraction of and isopropanol-mediated precipitation of RNA. RNA was stored in RNAase-free dH_2O at -70^0 C when it was required for assay work.

The RNA gel consisted of the following ingredients in the following amounts (for a 200 ml gel): 1) 2 g of agarose (FMC Bioproducts); 2) 20 ml 10x running buffer [200 mM MOPS (Sigma), 50 mM NaAcetate (Quality Biological, Inc.), 10 mM EDTA (Quality Biological, Inc.)]; 3) 3.6 ml Formaldehyde (Mallinckrodt). Resolution of RNA was achieved using 200 V, with the running time varying from 2 to 4 hours. Blotting was conducted using nitrocellulose paper (Schleicher Scheull) and transfer was left to occur overnight. After transfer RNA was cross-linked to the nitrocellulose paper using a UV cross-linker (Stratagene). Hybridization occurred in a hybridization cylinder. The blot paper was sufficiently soaked in hybridization buffer (178 ml dH_2O, Dextran [100 g in 200 ml dH_2O] (Amersham Pharmecia), 400 ml Formamide (Fluka Chemika), 200 ml 20xSSC (Roche), 10 ml 2 M Tris, pH7.4 (I/LT), 10 ml 100x Denhardt's (Research Genetics), 2 ml Salmon Sperm DNA (I/LT)) before the radioactive probe was added to the cylinder.

The probe was prepared by using Ready-To-Go DNA Labeling Beads (Amersham Pharmecia): briefly, after mixing 100 nM of DNA with dH_2O to bring the total volume up to 45 µl, ^{32}P-CTP (Amersham Biosciences) was added (5 µl) along with a DNA bead; the probe was then incubated at 37^0 C – the incubation time depended on the amount of labeling that was desired. Labeled probe was purified through EtOH precipitation: labeled DNA was phenol-chloroform extracted, after which it was incubated in dry ice with NaAcetate and 100% EtOH; the mixture was then microcentrifuged, the supernatant was discarded, and then the DNA pellet suspended in 100 µl of dH_2O. The labeled probe was then added to the following mixture: 1) 1.3 ml of dH_2O; 2) 200 µl Salmon Sperm; 3) 50 µl of 10N NaOH (Mallinckrodt). After probe addition, the following ingredients were then added, to complete probe preparation: 1) 140 µl of 2M Tris, pH7.5 (I/LT); 2) 500 µl of 1N HCl (Mallinckrodt). This entire mixture was then added to the cylinder containing the blot paper that had been soaked through with hybridization buffer. The cylinder containing the blot was left overnight at 42^0 C.

After probe annealing was allowed to occur, the blot was washed in the following way: 1) 3 washes at room temperature using 0.1% SDS (I/LT)/2x SSC at 15 min/wash; 2) 1 wash at 65^0 C using 0.1% SDS/0.1x SSC for 15 min. The blot was then wrapped in

saran wrap and left exposed to Kodak film at -70^0 C, to allow for autoradiography to occur.

Assay for protein

For protein extraction cells were first harvested in culture through culture-dish scraping in ice-cold PBS (scraping done on ice; cell-culture medium first removed before addition of PBS). Following transfer to a 15-ml conical tube, the cells were then centrifuged (5 min at 1200 rpm). After discarding the supernatant, the cell pellet was broken up and cells suspended in ice-cold PBS. The cells were then transferred to an eppendorf, which in turn was spun down to obtain a cell pellet (5 min at 13 Krpm [microcentrifuge]). Cells were lysed in buffer consisting of the following ingredients: 1) 0.5% 2M Tris; 2) 3% 5M NaCl (Digene); 3) 1% 500 mM EDTA; 4) 1% Triton (Sigma); 5) 10% Glycerol (I/LT); 6) 2 mM Vanadate (Sigma). Cells were left on ice for 5 min to allow cell lysis to reach completion, at which point the released material was spun down to remove cell debris (5 min at 13 Krpm). The supernatant was transferred to a fresh eppendorf, which was then stored at -70^0 C until it was required for protein resolution through an SDS gel.

Protein gels were made using the following ingredients: for the stacking gel (4.5%) – 1) 0.9 ml Acrylamide/bis (BioRad) (30%/0.8%); 2) 1.5 ml Buffer solution 1x; 3) 0.06 ml 10% APS (BioRad); 4) 0.01 ml 100% Temed (BioRad); 5) 3.6 ml H_2O/ for the resolving gel (12%) – 1) 3.6 ml Acrylamide/bis (30%/0.8%); 2) 2.25 ml Buffer 1x; 3) 0.063 ml 10% APS; 4) 0.012 ml 100% Temed; 5) 3.15 ml H_2O. The buffer for the stacking gel consisted of the following: 1) 0.5 M Tris; 2) 0.4% SDS; adjusted to pH of 6.9. The ingredients for the buffer used in the resolving gel: 1) 1.5 M Tris; 2) 0.4% SDS; adjusted to pH 8.8. Separation of proteins in the gel was achieved at a voltage of 200 V, using a gel apparatus from (BioRad). Proteins were then transferred to nitrocellulose paper using the same voltage and a transfer apparatus from the same company. The transfer buffer consisted of the following: transfer buffer: (1 L) 100 ml of Methyl EtOH (Fisher Scientific), 3.2 g Tris (I/LT), 14.4 g Glycine (ICN Biomedicals, Inc.). The blot was blocked in a solution of milk (5% milk in PBS with 1% BSA [Sigma]) for 1 hr (or in some cases overnight). Following an overnight exposure to the primary Ab, the blot was washed 4x in PBS/Tween-20 (Sigma) (0.1%). Exposure to the secondary Ab was for 30 min, followed by 3 washes in the PBS/Tween-20 solution. The blot was soaked in Pierce

SuperSignal solution, briefly dried and wrapped in Saran Wrap, and then exposed to Kodak film for different times at room temperature.

Polysome analysis

Cell extract was prepared in the following way: 1) cells were treated with 90 μg/ml of cycloheximide (Sigma) for 10 min at 37^0 C to chemically 'freeze' ribosomes on mRNA; 2) after a PBS wash (containing the same concentration of cycloheximide), cells were trypsinized using a trypsin solution (I/LT) containing the same cycloheximide concentration (5-min treatment time, or until cells were suspended in solution); 3) suspended cells were transferred to a conical tube on ice (ice-cold temperature employed to physically 'freeze' ribosomes on mRNA); 4) after spinning to collect the cell pellet, supernatant was discarded and the pellet transferred to an Eppendorf; 5) the cell pellet was stored in -70^0 C until it was used for fractionation. For cell fractionation cells were thawed on ice and then re-suspended in 200 μl of Reticulolysate Standard Buffer (RSB) [10 mM NaCl, 10 mM Tris(HCl) pH7.4, 15 mM $MgCl_2$]; cell lysis was accomplished through lysis buffer (10% Triton, 10% Deoxycholate in RSB), added to the cells in RSB at 1/10V; lysis was left to occur on ice (15 min), after which an equal V of Polysomal Buffer (PB) [25 mM Tris(HCl) pH7.4, 10 mM $MgCl_2$, 25 mM NaCl, 0.05% Triton X-100, 0.14 M Sucrose (Sigma), and Heparin (Sigma) (500 μg/ml)] was added to the lysate; the extract was then spun down to sediment membrane material. The supernatant was immediately layered on top of the sucrose gradient solution.

The sucrose gradient solution was made using a Sucrose-gradient Maker (VWR Scientific). The buffer used for both solutions consisted of the following ingredients: 1) 25 mM Tris(HCl) pH7.4; 2) 25 mM NaCl: 3) 5 mM $MgCl_2$; 4) Sucrose. The two sucrose solutions contained sucrose at a concentration of 5% and of 70%. The solutions were mixed through the Maker to make a continuous solution ranging continuously in concentration from 5% to 70% (top to bottom of tube).

After layering the extract on top of the sucrose-gradient solution, the sample was spun down to fractionate the extract into components, including polysomes and monosomes and ribosome subunits. (The principle behind this technique is that cell components can be resolved according to their respective masses (Meyuhas et al 1996): any particular component will sediment at a position within the tube that represents a

balance between the centrifugal force that 'pulls' it downward during centrifugation and the resistance offered by the viscosity of the sucrose concentration; the greater the mass of a component, the farther down it will sediment, so that cell material will fractionate into a range of components from the least mass (top of tube) to greatest mass (bottom of tube); mRNAs that are being actively translated are continuously associated with ribosomes – the greater the rate of translation, the greater the number of ribosomes that are 'attached' to an mRNA at a particular moment – so that the resulting complex of mRNA and ribosomes is itself a component of a particular mass; polysomes [actively translated mRNAs] are of greater mass than monosomes and ribosomal components.) The gradient solution with the separated components was then punctured at the bottom to allow for material to be released, a drip at a time. Fractions of material were collected by hand in eppendorf tubes that were held under the slowly dripping tube containing the gradient solution. The amount in each fraction was initially and arbitrarily chosen to be 0.75 ml – based on results gathered by other research groups using this technique, it was concluded that 16 fractions from the 12 ml gradient solution containing the fractionated cell extract would be adequate to resolve different polysome complexes and monosome complexes and ribosomal components. Stringent resolution of polysomes of differing masses was not required, and so the amount for fractions collected from the bottom half of the gradient solution was left at 0.75 ml (the first 8-10 fractions). Through trial and error, it was determined that a smaller amount for fractions collected from the top half of the gradient solution would allow for resolution of monosomes and ribosomal components; therefore the amount chosen was eventually settled at 0.15 ml.

Fractions were analyzed through the use of a spectrophotometer (Pharmecia Biotech), at an absorbance of 260 nM.

Figures and Legends to Paper 2

Fig 1 The Nucleolin protein level remains constant after serum depravation and during serum rescue. The same serum-starvation/serum-replenishment experiment was performed for both **A** LnCp cells and **B** Crl cells. The difference was in the length of the serum-deprivation period: 4 d for the Crl cells, 7 d for the LnCp cells. For both cell types the culture medium used for serum starvation was that normally employed for the cells, only without serum (0%). Cells were passaged and then 2 d later, serum starvation was begun after cells were washed 2x with PBS to remove any traces of FCS and then were placed in incubation with the FCS-depleted medium. Culture medium with 10% FCS was used for serum rescue. During serum rescue cells were taken out at the specified time points of incubation (1 cell culture/time point) for both RNA and protein extraction (see Materials&Methods for details of both protocols). For Northern blotting the RNA samples were run through a formaldehyde-agarose gel and then transferred to a nitrocellulose blot, which was then probed with a radioactively-labeled oligonucleotide representing the amino terminus of *nucleolin* cDNA. Protein samples were run through a 12% SDS gel and then transferred to a nitrocellulose blot, which was then cut in half before antibody incubation. The half that was determined through the aid of molecular markers to contain Nucleolin was incubated with an anti-Nucleolin antibody (Ab), and the other half with an anti-Actin Ab. The level of Actin is intended to be the loading control. 'Nuc' denotes Nucleolin and 'SR' denotes serum rescue.

Fig 1

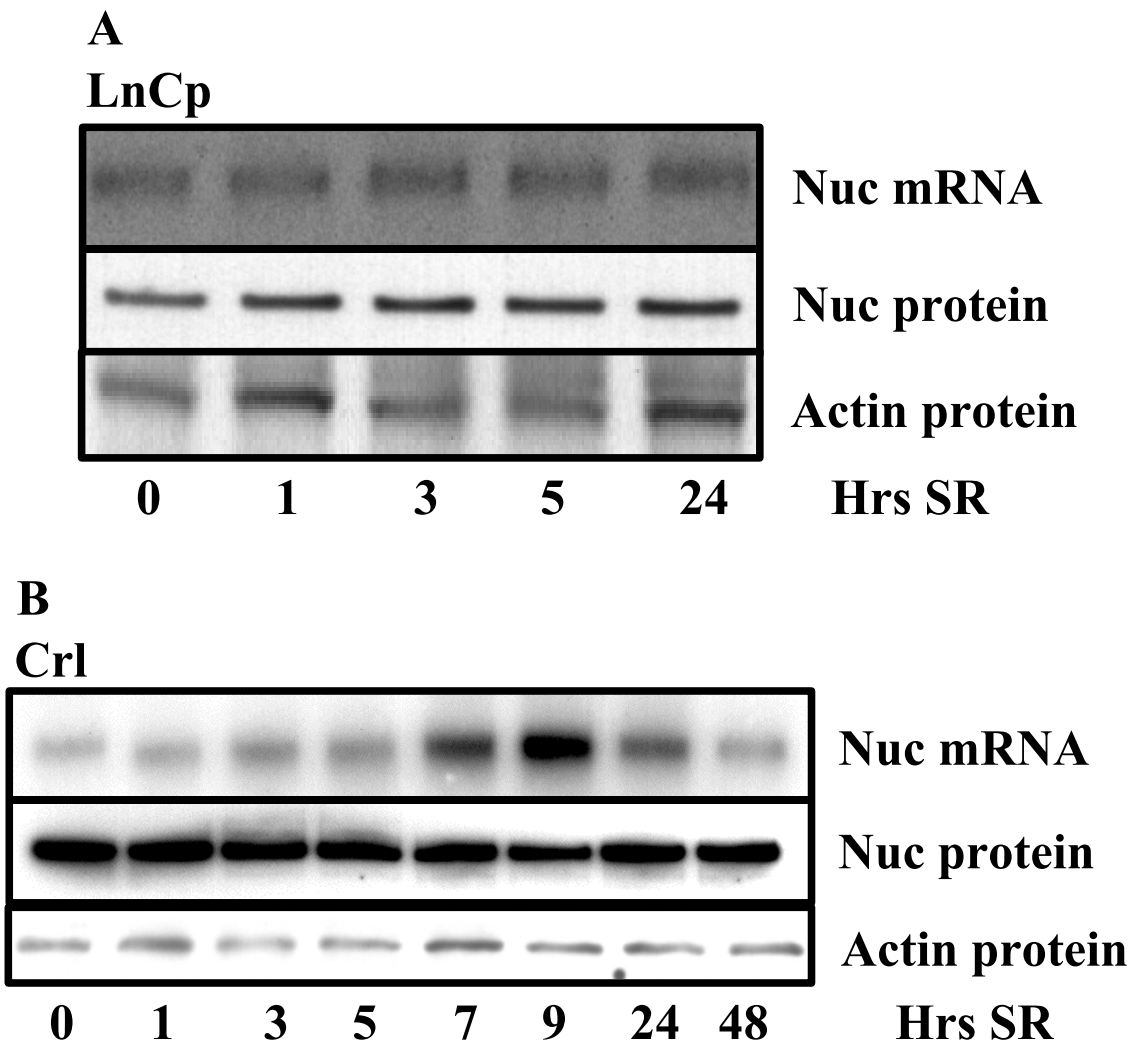

A
LnCp
Nuc mRNA
Nuc protein
Actin protein
0 1 3 5 24 Hrs SR
B
Crl
Nuc mRNA
Nuc protein
Actin protein
0 1 3 5 7 9 24 48 Hrs SR

Fig 2 The stability of Nucleolin protein Asynchronously-growing Crl cells were passaged and a day later, were treated with cycloheximide (cycH) (35 μg/ml). Treatment times were as indicated. *Lane 1* control population to indicate that cell passaging had no effect on the level of Nucleolin protein; *lane 2* cycH treatment for 24 hr; *lane 3* treatment for 48 hr; *lane 4* 72-hr treatment; *lane 5* control population that was left untouched in incubation for the 3-day experiment, to show that culture conditions had no effect on the Nucleolin level. Myc was used as a positive control to show the efficacy of cycH (see text of paper).

Fig 2

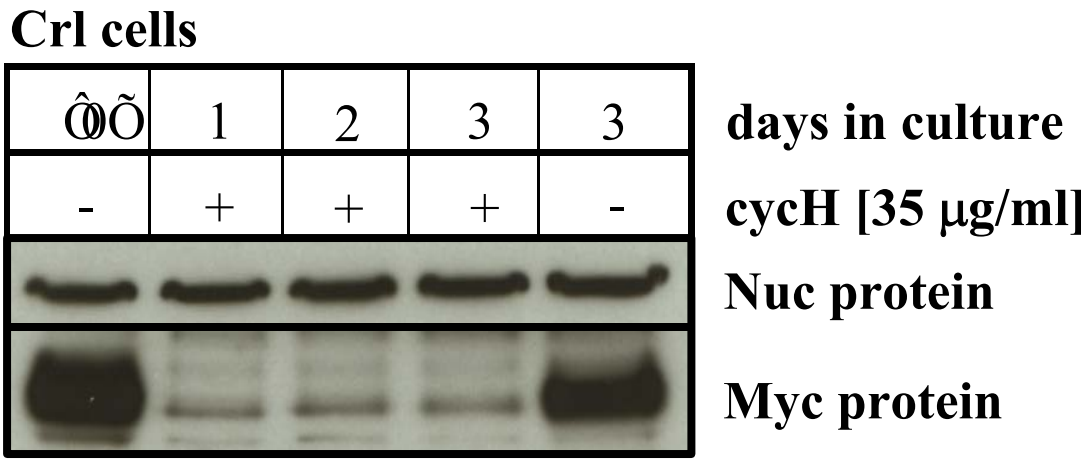

Crl cells
ÔØÕ
1
2
3
3
days in culture
-
+
+
+
-
cycH [35 μg/ml]
Nuc protein
Myc protein

Fig 3 The level Nucleolin protein is not affected by rapamycin. Asynchronously-growing **A** Crl cells and **B** LnCp cells were treated with rapamycin (0.2 μg/ml) for the designated amounts of time. Before rapamycin treatment, cells had been left in incubation for one day post-passaging to allow time for the cells to settle in culture. We then replaced the medium used for passaging with rapamycin-containing culture medium. Cells were harvested for protein extraction and a Western blot experiment was performed. The level of Myc was used as a positive control to indicate the efficacy of rapamycin, since translation of Myc mRNA has been documented to be lowered by rapamycin (see text). The level of Tubulin is meant to determine loading error.

Fig 3

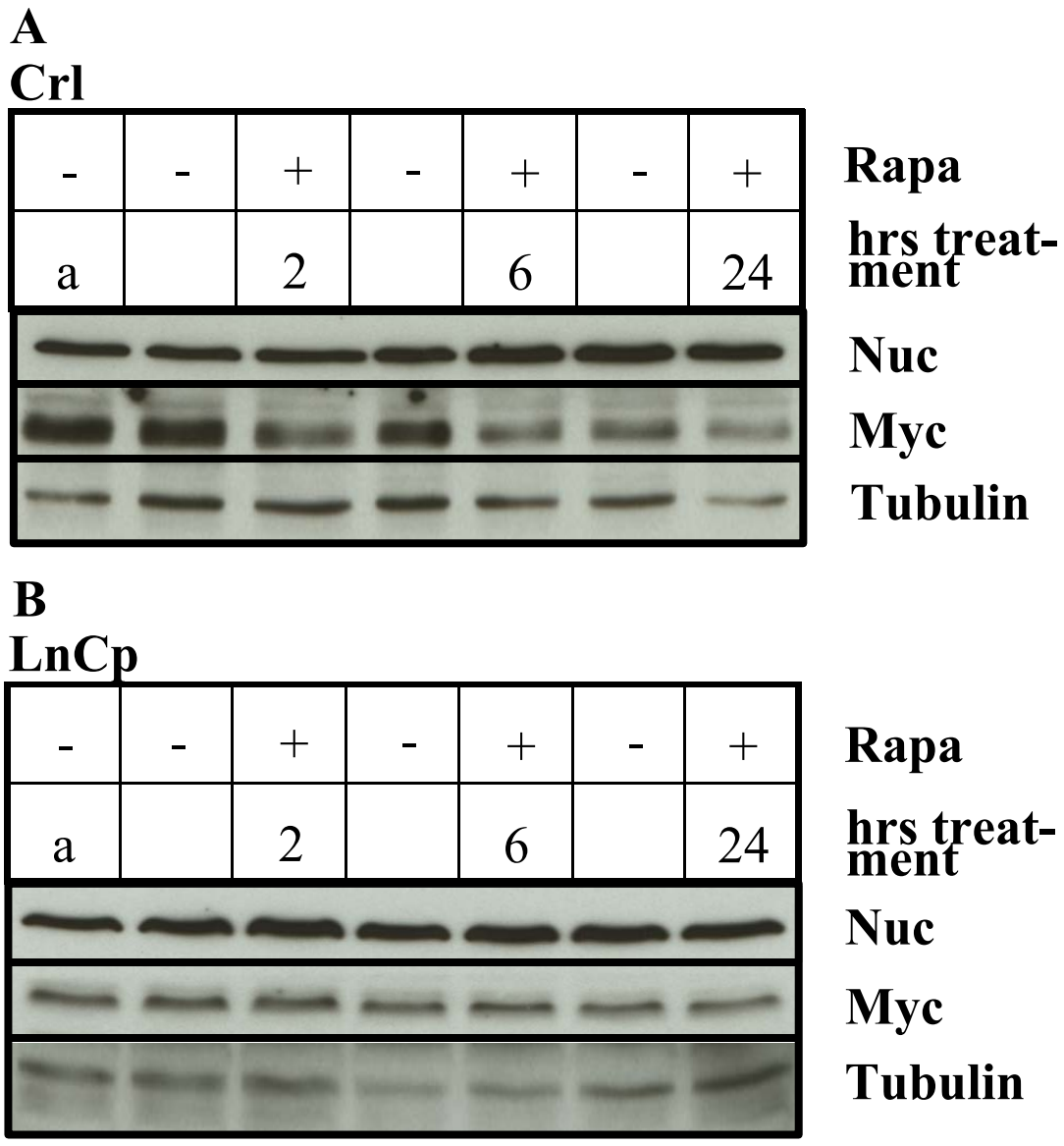

A
Crl
- - + - + - +
Rapa
a 2 6 24
hrs treat-ment
Nuc
Myc
Tubulin
B
LnCp
- - + - + - +
Rapa
a 2 6 24
hrs treat-ment
Nuc
Myc
Tubulin

Fig 4 A short period of rapamycin treatment of Crl and LnCp cells does not lead to polysome loss from translated mRNA's. Asynchronously-growing **A** Crl cells and **B** LnCp cells were treated with rapamycin for 2 hrs. The cells were then harvested for extract preparation. The extracts were to be analyzed through a sucrose-density gradient. The sucrose concentrations varied continuously from 5% to 70%. After the extracts were layered on top of the gradients (The extracts from both cell types were separately analyzed.), the gradients were centrifuged. Fractions were then collected for spectrophotometric analysis at an absorbance of 260 nm (for more details, see Materials&Methods). Both graphs represent a merging of data. Each point represents a spec reading of a fraction.

Fig 4

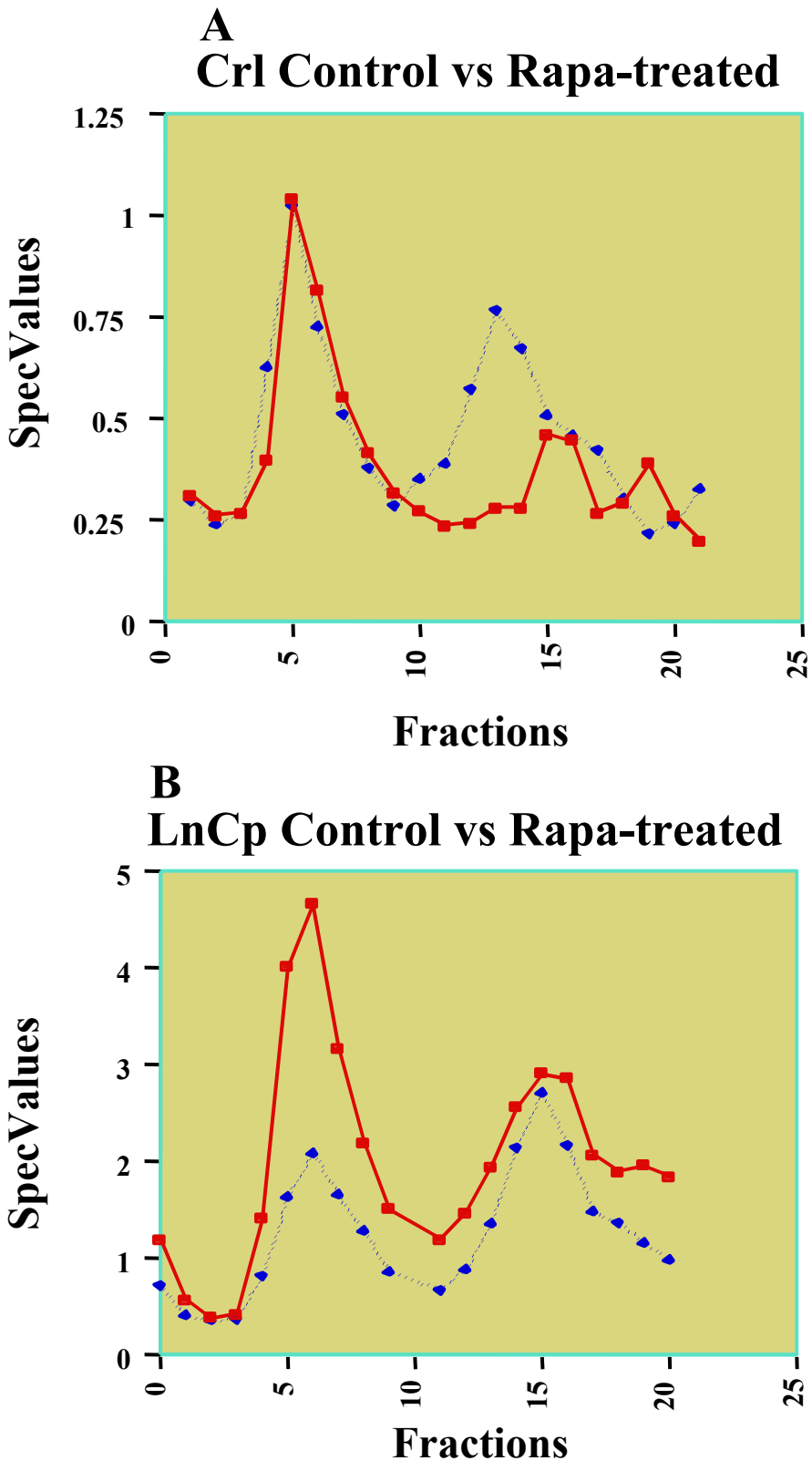
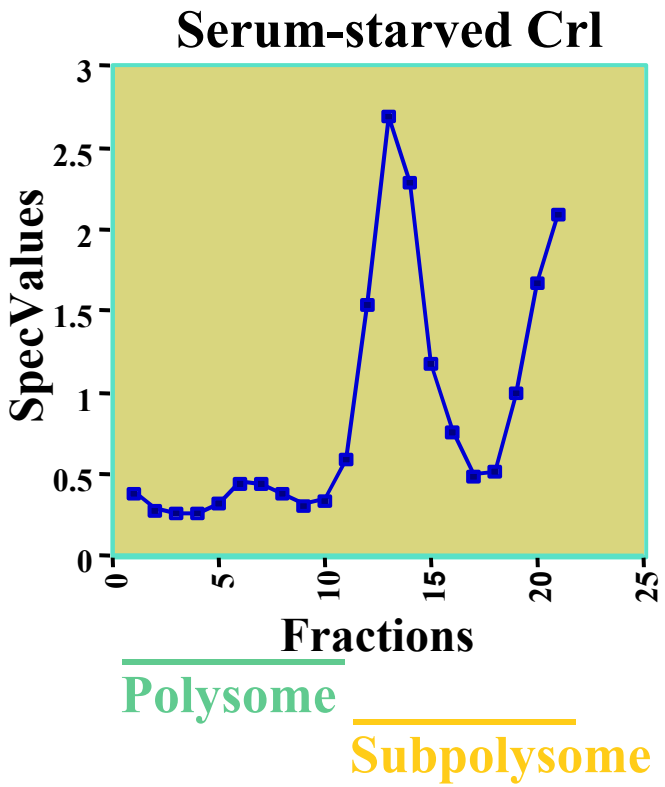

A
Crl Control vs Rapa-treated
SpecValues
1.25
1
0.75
0.5
0.25
0
0
5
10
15
20
25
Fractions
B
LnCp Control vs Rapa-treated
SpecValues
5
4
3
2
1
0
0
5
10
15
20
25
Fractions

Fig 5 The Nucleolin protein is synthesized in the absence of serum. SG analysis of serum-starved and serum-rescued Crl cells. **(a)** Crl Nucleolin synthesis at the end of the serum-depravation period **(b)** Nucleolin synthesis during serum rescue. The serum-starvation/serum-rescue experiment was the same as that conducted before (see Fig 1). Extract preparation for SG analysis was undertaken as described in Fig 4, and the same 5%-70% sucrose gradient was employed.

Fig 5

(a)


Serum-starved Crl
SpecValues
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
Fractions
Polysome
Subpolysome

(b)

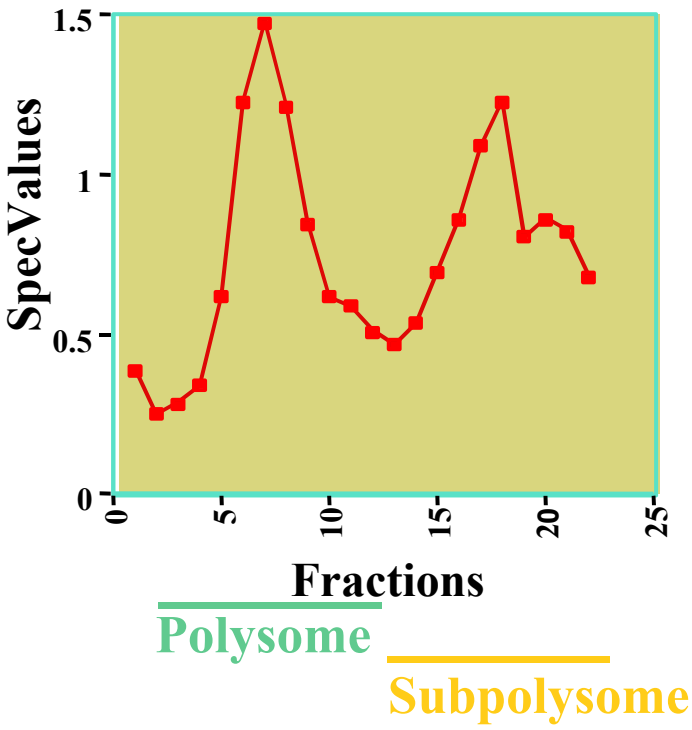

Crl 4hr Post-SR
SpecValues
1.5
1
0.5
0
0
5
10
15
20
25
Fractions
Polysome
Subpolysome

Fig 6 During serum rescue there is no concomitant degradation of Nucleolin protein with its synthesis. Crl cells were passaged, and 2 days later serum starvation was begun. For experimental cultures, after 4 hrs of serum rescue (serum re-stimulation of the previously serum-starved cells), cells were treated for 1 hr with cycH (45 μg/ml) or for 2 hr (in both cases with serum). Cells were then harvested for protein extraction. Shown is a Western blot to assay for Nucleolin expression. Myc expression was used as a positive control to show the efficacy of cycH. *Lane 1* cells at the end of serum starvation – a control population to show that, as demonstrated before, the cells enter quiescence (indicated by the disappearance of Myc protein); *lane 2* control population to show that the cells are responding as expected to serum (indicated by the upregulation in Myc protein synthesis); *lane 3* & *lane 5* control populations that had been serum-starved, then were serum-rescued and simultaneously treated with drug vehicle for 1hr and 2 hr, respectively; *lane 4* & *lane 6* experimental populations that were treated as described above (with cycH); *lane 7* control population to show that the 6 hr in culture during serum rescue had no adverse effect on the expression levels of Nucleolin and of Myc.

Fig 6

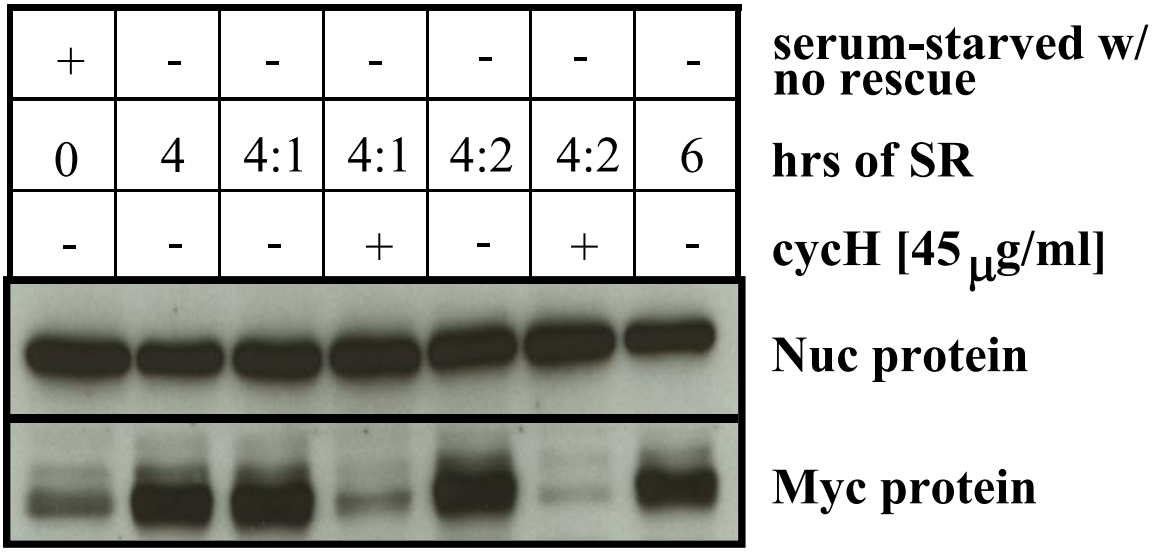

+ - - - - - -
serum-starved w/ no rescue
0 4 4:1 4:1 4:2 4:2 6
hrs of SR
- - - + - + -
cycH [45 μg/ml]
Nuc protein
Myc protein

Fig 7 A possible model of how Nucleolin protein may regulate its own synthesis See text in Discussion for more detailed examination of model.

Fig 7

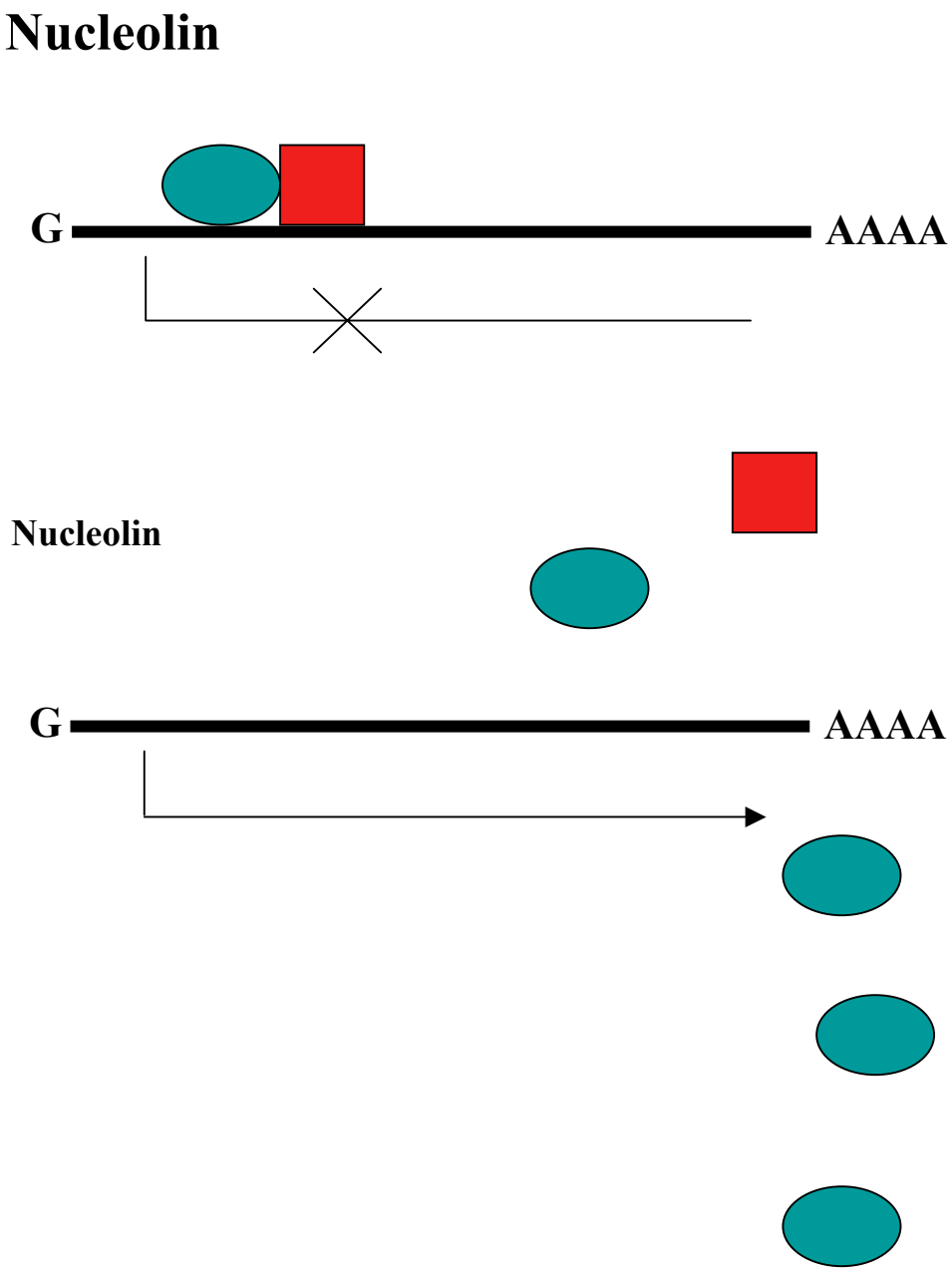


Fig 8A & 8B Ribosome biogenesis is not affected in the Myc-/- cell line. 8A Asynchronously-growing Rat1a and Myc-/- cell lines were harvested, and extracts were made for sucrose-gradient (SG) analysis, as described in Materials&Methods. Extracts were layered on top of a 5%-70% sucrose gradient, which was then spun using Sorval centrifugation. Fractions were then collected and read through a spectrophotometer. **8B** Both Rat1a & Myc-/- cell lines were serum-starved. After passaging of cells and allowing them to adhere to culture dish (1 day later), cells of both types were washed 2x with PBS to remove all traces of FCS. Then culture medium (DMEM) with 0.5% serum and insulin (100 nM) was placed in culture. After a 48-hr incubation, cells were harvested and extracts made for SG analysis. *Top half,* SG analysis of serum-starved Rat1a and Myc-/- cells. The data are merged. *Bottom half,* Cells that had been serum-rescued (4 hr in DMEM with 10% FCS) were harvested for extract preparation. The data from Rat1a and Myc-/- cells were derived through SG analysis and are merged in this figure.

Fig 8A

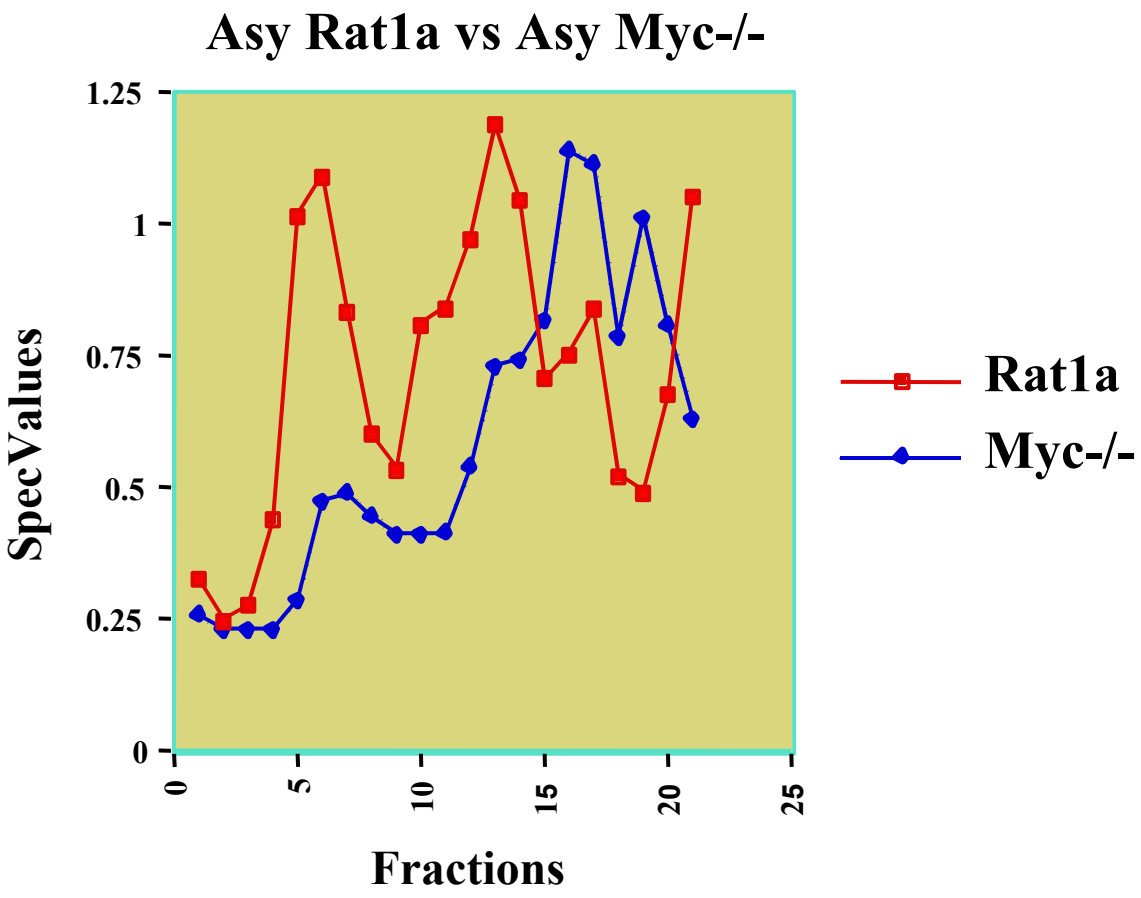
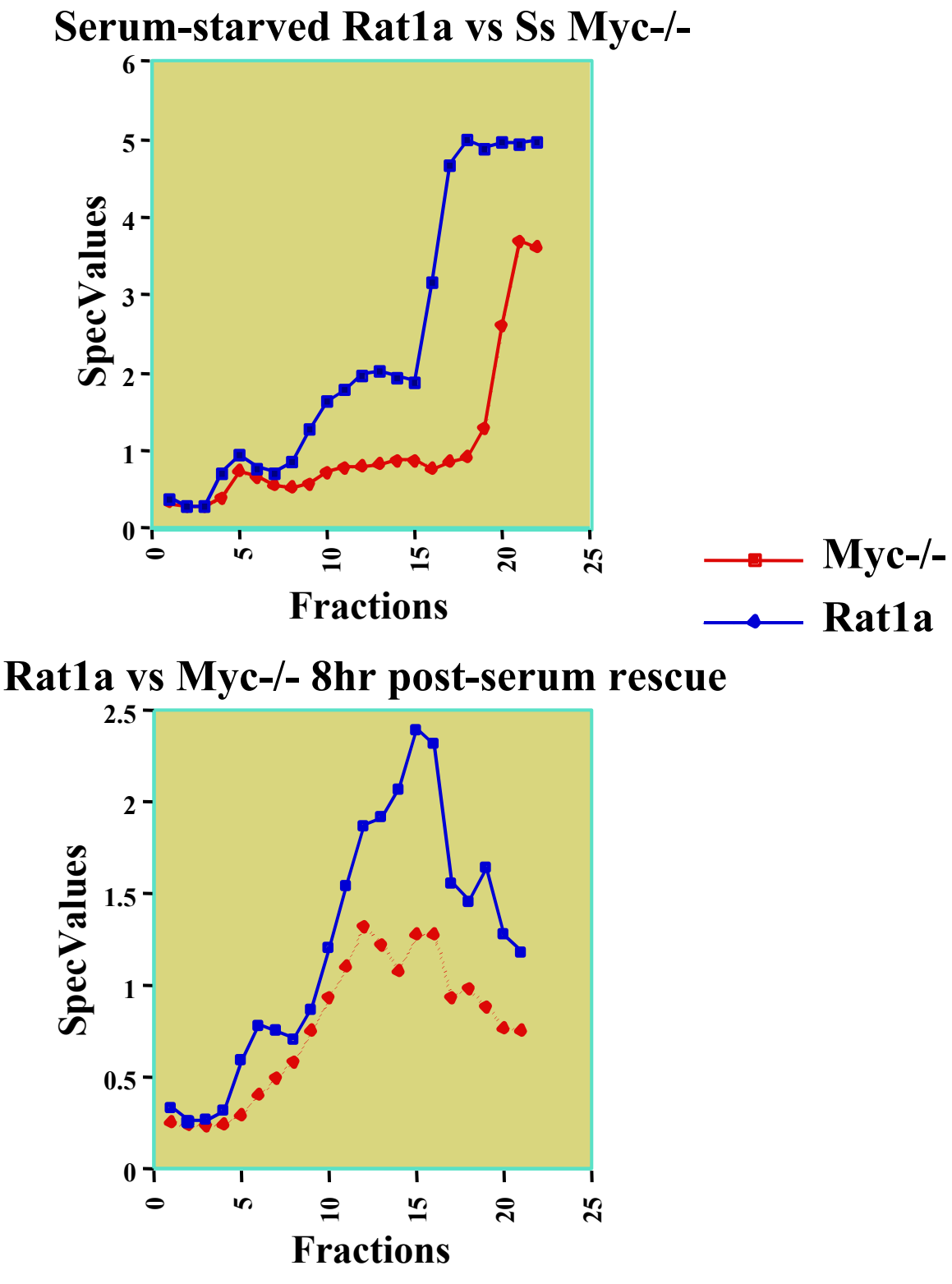

Asy Rat1a vs Asy Myc-/-
SpecValues
1.25
1
0.75
0.5
0.25
0
0
5
10
15
20
25
Fractions
Rat1a
Myc-/-

Fig 8B


Serum-starved Rat1a vs Ss Myc-/-
SpecValues
Fractions
Myc-/-
Rat1a
Rat1a vs Myc-/- 8hr post-serum rescue
SpecValues
Fractions

RESULTS

A discrepancy exists between the expression profile of Nucleolin protein and of Nucleolin mRNA In previous work we found that the level of Nucleolin mRNA remained constant in a prostate metastatic cell line (LnCp) throughout a particular condition (replicative quiescence induced by serum starvation) and through a change from that condition to a completely different condition (cell proliferation induced by serum) (Fig 1A). This constancy is in contrast to the mRNA pattern exhibited by a normal prostate cell line (Crl): downregulation in the mRNA level during quiescence and upregulation following serum addition (Fig 1B). As expected, the Nucleolin protein level was constant in LnCp cells through the course of this specific experiment (Fig 1A). This may be due to continuous translation of the mRNA. However, to our surprise, the protein level also remained the same in Crl cells (Fig 1B). Because the mRNA dropped to a relatively low level after a serum-deprivation period, we had predicted a similar drop in the protein level. With the rise of mRNA after serum rescue, we had expected the protein level to rise in similar fashion. Our next step was to understand the basis for this disparity.

The Nucleolin protein is highly stable One possibility we considered to account for the presence of Nucleolin protein even after a serum-deprivation period was its stability. If Nucleolin were highly stable, a new steady-state level of the protein following translational shutdown would not be immediately obvious through an immunoblot assay. The serum-deprivation period for Crl cells consisted of 4 days. The period is short enough that high protein stability might account for what appears to be continuous translation of the protein. Therefore, we wanted first to get a rough estimate of Nucleolin stability. We treated asynchronously-growing cells with cycloheximide, a drug that inhibits translation, and assayed for the presence of the protein during several days of treatment. As can be seen in Fig 2, the protein remained at the same level even after a 3-Day exposure to cycloheximide. We assayed for Myc to make sure the drug was working. Myc has a half-life of approximately 30 min in cells during logarithmic-phase of growth (Ramsay et al 1986) and therefore should disappear after 1 day of cycloheximide treatment. We were unable to treat cells for a greater length of time because the cells began to die by Day 4. It is possible, therefore, that the half-life of

Nucleolin may be even greater than 3 days. Its stability may be such that the protein may remain for as long as 8 or 9 (or more) days after translational shutdown. This may partly, if not wholly, explain the constancy in the protein level during quiescence of Crl cells.

Nucleolin protein synthesis is not directly nutrient-dependent via TOR Despite a low mRNA level, the protein translated from that RNA may be maintained at the same steady-state level it has when mRNA synthesis itself is ongoing (along with concomitant translation). This can occur through selective enhancement of translation using the remaining amount of mRNA. If the cell required a constant amount of a protein, even in the face of low mRNA level, the cell might respond by using another stimulus to activate a signaling pathway that resulted in enhanced synthesis of that protein. Despite the absence of serum, cells in culture might use the stimulation provided by nutrients in culture medium like amino acids. Such a pathway does in fact exist and is mediated by TOR (target of rapamycin) (see review in Gingras et al 2001). Serum-starved cells that are in replicative quiescence might nevertheless be able to maintain their metabolic state by responding to TOR-mediated nutrient stimulation to make ribosomes and in this way to synthesize protein factors that are needed under all conditions and those that are required during quiescence. The same TOR-mediate pathway can also affect the stability of proteins: inhibition of this pathway can lead to premature degradation of certain proteins.

We assayed for TOR activity by treating both LnCp and Crl cells with rapamycin (Fig 3A&B). We subjected asynchronous populations of both cell types to this drug for different time periods, extending treatment up to a day. What would make interpretation of any change in the Nucleolin level following a 24-hr treatment difficult is that the ribosome machinery itself might be dismantled (Indeed, we did observe this effect [see discussion in the following paragraph].) and so make translational shutdown of any protein a moot point. Nevertheless, we wished to determine whether or not rapamycin could have an effect on Nucleolin synthesis, and we reasoned that a day's worth of treatment should be sufficient to detect this. It has already been documented that Myc translation is partly inhibited by rapamycin (West et al, 1998), and so we used Myc level as our positive control. As can be seen with Crl cells, Myc level was lowered after treatment (Fig 3A), indicating that the drug was efficacious. Interestingly, the level

appeared to be unaffected in LnCp cells (Fig 3B). This could mean that rapamycin was inactive in this particular culture, although we treated both cell types at the same time so that drug inactivation while in storage is an unlikely explanation. Another explanation could be that LnCp cells carry a rapamycin-resistant TOR. We did not test for this. Our primary aim was to determine what effect, if any, rapamycin would have on the Nucleolin level. In any case, a rapamycin-resistant TOR is probably active and functional even under a nutrient-deprived condition. The data show that the Nucleolin level was not affected in both cell types (Fig 3A&B). At this point, however, it is difficult to conclude with certainty that rapamycin does not negate the specific translation of Nucleolin mRNA since it is possible that while TOR was in fact rendered inactive following treatment, the stability of Nucleolin might nevertheless be unaffected. As shown earlier, Nucleolin is highly stable. So even with selective inhibition of Nucleolin synthesis, an immunoblot would show the same steady-state level of Nucleolin that untreated cells possess. For this reason we decided specifically to assay for translation of Nucleolin mRNA following a serum-deprivation period (Fig 5). As discussed above, if there is selective translation of particular mRNA in serum-starved cells, whether through a TOR-mediated pathway or through another pathway using ingredients in culture medium besides serum for its stimulation, the particular proteins encoded by these mRNAs should remain at the same level even in the absence of serum.

One way to assay for ongoing translation is through ^{35}S-labeling of proteins. The protocol entails co-immunoprecipitating the protein under scrutiny, and here we came across a stumbling block since two different antibodies specific for Nucleolin that we used for our Western blots failed to precipitate the protein out of cell extract. For this reason we employed the sucrose-gradient technique as a substitute to detect translation. After fractionation of cell extract into polysomes and monosomes (along with ribosomal components), it is possible to extract RNA from fractions collected. mRNA analysis through a conventional Northern can then be conducted, and synthesis of any particular protein can be inferred from the association of its mRNA with the polysome fractions (inhibition of synthesis shown by lack of association).

One issue to be resolved first was whether or not rapamycin led to translation shutdown on a global scale in the experiment described above in which we treated Crl

and LnCp cells with rapamycin (Fig 3A&B). We used the sucrose-gradient technique to address this point. We prepared extracts from Crl cells (Fig 4A) and LnCp cells (Fig 4B) that had been treated with rapamycin for 2 hrs. This treatment time spared the polysome portion (Fig 4A&B). Polysome loss did occur in both cell types after a 24-hr exposure to rapamycin (data not shown). Therefore, at least at the 2-hr time point, the presence of Nucleolin was not due to selective enhancement of translation of its mRNA (see Fig 3A&B, lane 3). Such an enhancement might account for the constancy in its level after the 24-hr treatment. However, as discussed above, since Nucleolin is highly stable and certainly does not disappear after 1 day following inhibition of its synthesis, the Nucleolin that we could detect in the immunoblot -- Fig 3A, *1st row, lane 7* and Fig 3B, *1st row, lane 7* -- represented the level of Nucleolin that remained even after its synthesis had been turned off by rapamycin. The dramatic loss of polysomes that we observed in both cell types after 24-hr exposure to rapamycin makes it doubtful that translation of any protein was ongoing at this point.

It does not appear that translation of Nucleolin mRNA occurs after serum starvation We used the same sucrose-gradient technique to assay for Nucleolin synthesis at the end of serum starvation and during the period immediately following serum addition (4 hrs post-serum rescue [SR]). Extracts from both quiescent and serum-rescued cell populations were prepared on the same day, as were centrifugation of samples and collecting and reading of fractions. Therefore, we doubt that the drastic disappearance of the polysome portion in quiescent cells (Fig 5a) can be attributed to systematic error. We interpret the absence of polysomes to indicate that global-scale translation has shutdown. Another point of interest is what appears to be an accumulation of unemployed ribosomes (note the relative rise in the level of the monosome fraction) (Fig 5a). Disassembly of ribosomes did not appear to have occurred in quiescent cells. Instead, previously active ribosomes were themselves functionally quiescent, but on standby, so to speak, ready to work again as soon as the cells received the proper stimulus to direct their metabolic energy to the task of cell proliferation and cell growth (Fig 5b). The graphs suggest that if translation of any particular mRNA were ongoing, that instance of translation must be one that is either selectively enhanced or so efficient in the first place that it is unaffected by a lowering in the cell's metabolic energy. We are currently investigating this

possibility, although it appears that global translational shutdown has occurred. Therefore, despite the ongoing presence of nutrients in the serum-depleted culture medium, Nucleolin protein does not appear to be synthesized through a TOR-mediated pathway (unless nutrients are in fact a stimulus source but cannot stimulate the cells since the TOR-mediated pathway is non-functional in our Crl cells).

During the serum-rescue period Nucleolin protein is not degraded at the same time that new Nucleolin protein is synthesized One possible, albeit unlikely, explanation for protein-level constancy despite an mRNA rise is that while translation of the mRNA does occur, either the newly-synthesized protein is degraded as soon as it made or 'old' protein is degraded to maintain the protein at the same level. To test for this, we treated recently serum-rescued cells with cycloheximide and then assayed for Nucleolin immediately after treatment. It is the period immediately following serum addition that is in question, and so we attempted to detect Nucleolin in these treated cells. We reasoned that if Nucleolin protein were quickly degraded, we should observe a rapid disappearance of Nucleolin soon after cycloheximide treatment of the serum-rescued cells. We used Myc as a control to show the efficacy of cycloheximide; as Myc has a half-life of 30 min, we reasoned it should disappear after the 1-hr treatment. This is confirmed by our data (Fig 6, *third row*). However, Nucleolin maintained the same level (Fig 6, *second row*). Therefore, there is no concomitant degradation with synthesis of Nucleolin.

Nucleolin might participate in autoregulation of its own synthesis by prohibiting translation of Nucleolin mRNA when it -- protein -- is in excess Fig 7 is a hypothetical model. Nucleolin has been shown to be able to bind mRNA, its mode of action and effect depending on the particular mRNA to which it is bound. Therefore, we speculate that when Nucleolin is in sufficient supply, it may bind its own mRNA as well to inhibit translation. The word 'Nucleolin' is displayed in letters of two different sizes: the larger size in the top half of the figure is meant to signify an ample supply of the protein. In this particular condition, Nucleolin -- shown as the round molecule in bluish green -- binds cooperatively with a hypothetical factor -- shown in red. The mechanism of translation inhibition must be one that preserves the mRNA, since we found no evidence that the mRNA degrades during the period of serum rescue. Therefore, translational shutdown likely does not involve de-capping or de-adenylation of the mRNA, as is the case with

ARE-binding proteins that inhibit translation and de-stabilize the mRNAs that they bind. Translational shutdown mediated by Nucleolin may involve prohibition of translational elongation. When the Nucleolin level drops -- signified by 'Nucleolin' in smaller letter, in the bottom half of the figure -- the protein loses its affinity for its mRNA as a new equilibrium is achieved between binding to its mRNA and non-binding as the protein floats away. Because the hypothetical factor requires Nucleolin to interact with Nucleolin mRNA, it too loses its affinity for the mRNA under this condition. At this point translation can commence to produce new Nucleolin protein, until its level reaches the original point.

The slow growth of Myc-/- cells is not due to insufficient ribosome biogenesis, but rather to a slow rate of translation initiation As discussed above, sucrose-gradient analysis has the capacity to gauge ribosome biogenesis. We took advantage of this by analyzing asynchronous Rat1a and Myc-/- cells for their ability to make ribosomes. Myc-/- cells grow slowly (with extended G1 and G2 periods so that the average size of the cells remains the same as that of Rat1a cells). This phenotype may be due to lowered ribosome biogenesis, though cells of *min* Drosophila mutants – which are also defective in ribosome biogenesis – show a different cell-growth phenotype (Gallant et al 1996). Our data show the presence of 60S and 40S ribosomal subunits and of monosomes in Myc-/- cells (Fig 8A). However, the subunits do not appear to be active as 80S whole ribosome complexes in the Myc-/- cells. That the number of polysomes is less than that in Rat1a cells is consistent with this. Therefore, the deficiency in the Myc-/- cells in terms of their ability to grow at the normal rate appears to be due less to a deficiency in ribosome biogenesis and more to a defect in translation initiation. Myc-/- cells do make Nucleolin (our previous work). Therefore, if Myc targets the *nucleolin* gene in order for its protein to perform a rate-limiting step in cell growth, that step cannot be ribosome biogenesis but rather one in translation initiation, in which there is no documented evidence that Nucleolin is active.

The rate of ribosome biogenesis between Rat1 cells and Myc-/- cells is comparable In the previous section we assayed for ribosome biogenesis in asynchronous Myc-/- cells. From our data we conclude that under conditions of logarithmic growth, ribosome biogenesis is non-rate-limiting in this cell type. However, under sub-optimal growth

conditions, where ribosome activity is lowered (possibly along with ribosome assembly), ribosome biogenesis might then drop to such an extent that during re-entry into the growth and proliferative phase after cells are stimulated to grow, ribosome biogenesis might become rate-limiting. If this were the case, Nucleolin in its ability to facilitate ribosome assembly might be normally targeted by Myc to promote growth at its normal rate. Without Myc, there might not be enough Nucleolin to fulfill this particular activity. Therefore, we tested for this by performing sucrose-gradient analysis of serum-starved Myc-/- cells (and of serum-starved Rat1a cells for comparison). While it does appear that ribosomes were disassembled in these cells (Fig 8B, *top graph, in red*), ribosome components were synthesized at the same rate, but not to the same extent, as that shown in Rat1a cells after serum addition to the cells (Fig 8B, *bottom graph, in red*). Therefore, we conclude that even in sub-optimal growth conditions, Myc-/- cells can make ribosomes at a normal rate. Their slow growth appears to arise from their inability to use these ribosomal components to translate mRNAs at the same rate as translation in Rat1a cells.

DISCUSSION

In this paper we attempt to determine why the level of Nucleolin protein remains constant, both after a period of serum starvation and during serum rescue of cells. Although it has been reported by others that this level declines when cells are quiescent or somehow prohibited from growing, we cannot say that our finding represents an aberration of the prostate metastatic cell line that we worked with. We found this same constancy in cell lines derived from normal prostate tissue. Another point of interest where our finding differs from past results is that the protein did not increase in amount when we stimulated our cells to grow and proliferate. We attempted to address both issues as a means to understand Nucleolin biology. As we will discuss in further detail later on, the constant level of Nucleolin indicates a stringent regulation of this level, but also hints at its function since cells apparently require a certain amount always to be on hand for Nucleolin to perform its specific biochemical activity or activities.

There is ample documentation to show that Nucleolin directly participates in ribosome biogenesis. As ribosome biogenesis is ongoing in asynchronously-growing cells, it is not surprising that Nucleolin is always present. However, its presence during cell quiescence is puzzling. When cells cease to proliferate, Nucleolin has been reported to undergo self-cleavage (Chen et al 1991). We found no evidence for this using two different antibodies to Nucleolin and assaying for released parts of the protein through a conventional Western (data not shown). Self-cleavage may be specific to certain cell types. It may just as well depend on the means of inhibiting cell proliferation. No other study has investigated the effect on Nucleolin of completely depriving prostate cells in culture of serum.

Another indication we got that Nucleolin is always required is its stability. After three days of treating cells with cycloheximide, the level of Nucleolin did not visibly decline. As discussed in the Results section, we could not extend the treatment further since the cells began to die in great numbers on day 4. It is possible that there might be a quick and precipitous drop of Nucleolin beginning at day 4. However, assuming a gradual decline, we think that the Nucleolin half-life may be 4 days or an even greater time period (In other words, its complete disappearance in the absence of translation may occur on day 8 or on days after.). This high stability may account for its presence in

serum-starved Crl cells (our normal prostate cell line). The serum-deprivation period for these cells was 4 days. Assuming translational shutdown, the level may nevertheless show no immediate decline on the fourth day, given the stability of Nucleolin, since it would take time for a lower steady-state level to register through an immunoblot.

Another possibility we considered was that Nucleolin might be maintained not only as a result of its stability but also through TOR(Target of Rapamycin)-mediated synthesis of the protein. Our serum-starved cultures lacked serum but not nutrients like amino acids and vitamins that can be found in cell-culture medium. There is mounting evidence to show that TOR somehow senses nutrient availability to regulate translational synthesis of ribosomal factors (see review in Gingras et al 2001). So the protein-synthesis machinery, and therefore cell growth, depends in part on TOR, besides other intracellular signal components that sense growth factors. TOR is a protein kinase and one of its targets is S6K, which, in turn, phosphorylates the ribosomal protein S6. The latter phosphorylation event is nutrient-sensitive. A rapamycin-resistant S6K can phosphorylate S6 even when cells are deprived of nutrients. This and other evidence point to a crucial function of TOR in cell growth. For this reason we thought that Nucleolin may be synthesized during quiescence as a result of nutrients in the FCS-depleted medium that keep TOR in an activated state. This does not appear to be the case in our Crl and LnCp cells.

Constancy in the Nucleolin level suggests tight regulation of this level. Cells appear to require its constant presence even during cell quiescence when ribosome biogenesis is relatively inactive. Nucleolin has been reported to be multifunctional, although this picture of multifunctionality may be inaccurate since biochemical processes whose proper function has been attributed to Nucleolin may in fact be carried out by Nucleolin-like proteins rather than Nucleolin itself (see review in Ginisty et al 1999). These proteins harbor a similar tripartite structure as that of Nucleolin, but represent distinct molecular species. If cells require Nucleolin only for ribosome-subunit assembly, then its presence during cell quiescence indicates that cells are ready to engage in rapid ribosome biogenesis in ‘anticipation’ of growth re-stimulation; a delay that results from having to synthesize Nucleolin can be avoided in this way. We conducted polysome analysis of serum-starved cells through a sucrose gradient, and our data

showed that ribosomes that had been active in translation before serum starvation were not disassembled. They appeared to be held at bay. So the assembly of new ribosomes as cells are re-stimulated may be unnecessary for cell growth. However, to enter the first cell cycle after quiescence, cells may need to make new ribosomes (Volarevi´c et al 2000). Nucleolin that is already present and ready to go may contribute to the rapidity of this process. If it turns out that Nucleolin fulfills another purpose, this activity would have to be one that is needed during quiescence. As one possible scenario, Nucleolin may allow the cell to interact with its extracellular environment. Nucleolin has been localized to the cell surface, where it is proposed to bind Actin (Hovanessian et al 2000) and Laminin (Kleinman et al 1991).

The constancy of the observed level also suggests that our cells cannot tolerate a higher level of Nucleolin. We attempted to make a stable transfectant using the nucleolin gene under CMV regulation. Although it was relatively easy to make a stable transfectant with the expression vector, all attempts at stably incorporating nucleolin into the genomes of four different prostate cell lines (one of our normal prostate cells, and three prostate metastatic cell lines, including the LnCp cells) failed. We did not determine the kind of death that the cells went through since it would have been difficult to disentangle whatever we might have ascertained from the contributory effect of G418, in this case the means of selective pressure. Our attempts at inducing ectopic nucleolin expression also failed with three different inducible promoters.

It is tempting to speculate that a rise in the Nucleolin level above the permitted norm may cause cell death, possibly apoptosis. If this were the case, it would be equally intriguing to determine the consequences both of raising the Nucleolin level and of suppressing cell death. Is nucleolin an oncogene? It can be activated by the Myc protein. Although we demonstrated in previous work that Myc did not transactivate the endogenous nucleolin gene, we did not address the possibility that Myc can transactivate the gene under a particular circumstance. The research groups submitting the result that nucleolin is a Myc target did precisely this work – overexpressing ectopic myc. An area of investigation in Myc-target work focuses on whether or not genes that are activated by Myc are the same and only genes that are activated in Myc-associated tumors when Myc is at a high level (Cole and McMahon 1999). Because the E-box element to which the

Myc-Max complex binds exists at every gene (the estimate is 1 to 3 sites per gene) (see Schmidt 1999), it is possible that Myc in abundance may activate genes that it normally does not interact with. A high Myc level may lead to a higher rate of transcriptional initiation at genes, including those that it normally does not transactivate. This in turn may lead to a higher-than-normal amount of the proteins encoded by these genes. If this were true, one question to address would be whether or not overexpression of any of these genes independently of Myc is sufficient to mimic some, if not all, of the biological phenotypes exhibited by a cancer cell in which Myc is active at a high level. Loss of regulatory control of Myc can lead to apoptosis (see Prendergast 1999). The mechanism by which this occurs is currently unknown. It is possible that proteins that act downstream to Myc may mediate its effect(s) that induces apoptosis. This may account for our inability to construct a stable nucleolin transfectant that expresses nucleolin ectopically and constitutively at a high level.

Another possible reason that cells may be unable to tolerate high Nucleolin levels is that excess Nucleolin may have an inhibitory effect on rDNA transcription. When Roger et al (Roger et al 2002) injected Nucleolin into the nuclei of Xenopus oocytes, the group found that normal rRNA synthesis was disturbed: transcriptional elongation was abnormal, with irregular spacing between elongating complexes rather than the normal procession of complexes to indicate smooth, continuous transcription of an rDNA locus; in addition, the terminal balls at the 5' ends of nascent pre-rRNA that can be observed through EM photographs of Christmas Trees (An rDNA locus is transcribed at a constant rate with many elongating transcriptional complexes simultaneously progressing through the locus so that the transcribed unit has the appearance of a Christmas tree in an EM photo.), a terminal ball being an pre-rRNA processing complex, were missing or abnormal-looking. Therefore, excess Nucleolin may actually result in retarding cell growth.

If the regulatory mechanism that sets the Nucleolin level does not permit a higher amount, the conclusion we draw from this is that cells have enough for all its various activities, including ribosome biogenesis. This implies that Nucleolin is in excess, and excess Nucleolin may itself suppress further translation of Nucleolin mRNA (Fig 7). This might occur directly as a result of Nucleolin binding to its mRNA. It has been

shown that Nucleolin can bind RNA species besides rRNA: it binds the Amyloid Precursor Protein mRNA and the IL-2 mRNA. Nucleolin could bind its mRNA as it is being exported from the nucleus, possibly even facilitating that export. It could also bind the mRNA once it is in the cytosol. Just as possible could be its binding the mRNA at the nucleolus. Myc mRNA has been localized to the nucleolus (Bond and Wold 1993), and so there is precedence for this sort of localization. By binding the Nucleolin mRNA along with an interacting protein(s) at an adjacent binding site(s), Nucleolin might contribute to translational suppression.

Nucleolin in excess also implies that it is not rate-limiting for any particular process in which it participates. Regarding its multifunctional nature, there is no study to show that different intracellular pools of Nucleolin exist and that each is maintained at a certain concentration. If they were in fact to exist, each pool might be reserved for a particular activity. As a possible example, Nucleolin was found in the FMRP-associated mRNP particle (FMRP is encoded by FMR1, the gene whose loss of expression is the cause of Fragile X syndrome [Ceman et al 1999].); although it is possible that cytoplasmic Nucleolin is inserted into this particle, it may be as likely (Ceman et al 1999) that the complex incorporates nucleolus-localized Nucleolin as it forms, given the localization of a small pool of FMRP proteins in the nucleolus (see Discussion in Ceman et al 1999). Because Nucleolin is in excess, a drop in the concentration of any pool would lead either to Nucleolin re-distribution among the pools to restore balance in Nucleolin concentration among the pools, or to Nucleolin transport from a storage center to the depleted pool. This hypothetical storage center may or may not coincide with the nucleolus.

Myc appears to target a rate-limiting step in cell growth. Ectopically-expressed Myc can effect cell growth within the same cell cycle in which the ectopic gene is expressed. It has been proposed that that rate-limiting step is a step in ribosome biogenesis. Therefore, the status of the nucleolin gene as a Myc target as been rationalized on this basis, that it is activated by Myc to produce new Nucleolin that will facilitate making more ribosomes so that the cell can grow at a faster rate. However, as discussed above, our data suggest that Nucleolin is likely not rate-limiting for any of its activities, including ribosome-subunit assembly. Therefore, if targeting of nucleolin by

Myc were real and a consistent phenomenon in Myc-associated cancers, then the activity for which Nucleolin were recruited by Myc must be one that it normally would not facilitate, but could participate in by virtue of some property it possesses, like its RNA-binding capacity. Even if Nucleolin under normal physiological conditions were to bind with low affinity a particular factor X, whether RNA or protein, Nucleolin might bind X tightly if it were in excess and if the cell could be made to tolerate excess Nucleolin. Our study of the Rat1a and Myc-/- cell lines through sucrose-gradient analysis supports this idea (Fig 8A&B). Under normal cell-culture condition (that in which asynchronously-growing cells grow and divide), both cell types are able to make ribosomes (Fig 8A). During serum starvation the Myc-/- cells appear to disassemble their pre-existing ribosomes (Fig 8B, top half). Nevertheless, during serum rescue the cells clearly display the ability to assemble new ribosomes, though to an extent that is lower than that demonstrated by the Rat1a cells (Fig 8B, bottom half). The important point is that the kinetics of ribosome biogenesis appears to be comparable between the two cell types, indicating that the absence of Myc does not impair the rate of ribosome biogenesis. The more likely explanation is that Myc targets translation initiation, whether by the transactivation of genes encoding initiation factors or by a post-transcriptional process that involves those factors. We have shown in previous work the presence of Nucleolin mRNA in the Myc-/- cells. Because these cells can synthesize Nucleolin independently of Myc, ribosome biogenesis is unaffected in this cell type. If a specific cellular context could be found in which Nucleolin were at a level lower than its usual amount (that which is found in logarithmically-growing cells), it would be interesting to determine whether or not this correlated with a lowering in the rate of ribosome biogenesis. It may be possible that in this circumstance Myc in excess may directly target nucleolin to accelerate ribosome biogenesis and thereby promote both cell growth and cell proliferation. Once the amount of Nucleolin reached its normal steady-state level, the cell would no longer use Myc for the purpose of transactivating nucleolin. As previously mentioned, research groups have documented a low Nucleolin level after specific treatments of their cells (Ohmori et al 1990; Konishi et al 1995; Westmark and Malter 2001; Miranda et al 1995; Mehes and Pajor 1995; Konishi et al 1995; Murakami et al 1991). In such systems it would be interesting to see what sort of effect the expression of

ectopic myc would have not only on ribosome biogenesis but more specifically on the transcriptional activity at the nucleolin locus.

In conclusion we believe that a constant level of Nucleolin reflects a requirement of cells to maintain its strict presence. This may or may not relate completely to ribosome biogenesis. Since Nucleolin participates in various different cellular processes, its presence and stability may be a means by which the cell ensures its active participation in these processes at all times. Our results also show that reliance on mRNA data alone may be deceptive; a discrepancy between protein expression and mRNA expression should always be taken as a possibility. In fact precedence for such a disparity in Nucleolin expression does exist (Maridor et al 1990): there is a clear separation between mRNA expression and protein expression during chicken embryogenesis and in the adult stage of chickens.

SUMMARY DISCUSSION

This discussion will proceed as follows: 1 a very brief summary and examination of the two papers will be presented; and, 2 the Nucleolin field will be scrutinized, with suggested directions for future work.

The one point that can definitely be taken away from our work is that regulatory mechanisms that are directed towards mRNA and those that are directed towards protein are distinct and may operate independently of each other. However, we believe that regarding Nucleolin biology, the mechanisms serve a common purpose, which is to ensure the cell a constant and ready supply of Nucleolin. Although mRNA regulatory controls appear to be lost in LnCp cells, that loss hints at the importance of Nucleolin in cell proliferation. Unless Nucleolin mRNA can serve a non-coding purpose, we assume its constancy in the metastatic cell line points to a need to have a constant amount of Nucleolin protein to perform its various different tasks as the cell grows and divides. The high stability of Nucleolin protein is consistent with this notion and attests to its relevance in every phase of the cell cycle. As crucial as Nucleolin is, however, its maintenance at a certain level may be just as significant, since a higher amount or hyperactivity of the protein may lead to growth-related malfunction like cancer or the loss of coordination among its different biochemical activities with fatal consequences to specific aspects of cell homeostasis. Future work for us to undertake include the following: 1 specifying the signaling pathway(s) that lead to transcription of the *nucleolin* gene and the transcription factors involved; 2 determining how aberrant transcriptional regulation can lead to Nucleolin mRNA constancy like the kind that we observed in LnCp cells; 3 pinpointing the mechanism by which Nucleolin mRNA can be held on standby until the cell uses it to synthesize Nucleolin protein; 4 determining how the cell senses that there is an adequate amount of Nucleolin and how this information is then relayed to the regulatory device keeping the mRNA in check transcriptionally; 5 determining the mechanism enabling Nucleolin to possess high stability; 6 determining how the cell maintains strict regulation of the Nucleolin protein level; 7 determining the consequences of Nucleolin over-abundance and what particular activities that have been attributed to Nucleolin are affected (i.e., whether or not the rate of an activity increases, whether or not an activity is in fact inhibited when there is too much Nucleolin).

The only way to definitively assess the importance of Nucleolin is through gene deletion, whether in a knockout mouse or in culture cells through homologous recombination. If the absence of *nucleolin* is lethal, a conditional knockout system might be more suitable, like *nucleolin* under Cre-Lox regulation. This can be used to determine the role of Nucleolin at different times of development, as well as in different tissues. In addition, Nucleolin function in different organs that is specific to the adult stage can be gleaned.

One research area of particular interest that may benefit from conditional knockout work of *nucleolin* is the relation of cell proliferation to cell growth. It may be fruitful to compare the regulation of Nucleolin in two 'types' of cells, one that coordinately grows and divides, like fibroblasts, and a post-mitotic cell that continues to grow, like neuroblasts that are becoming fully differentiated. One particular study (Volarevi´c et al 2000) found that as a cell enters and progresses through G1, it can and will use ribosomes that were inherited from the dividing cell that produced it and a sister cell. However, in order to progress through the cell cycle, it must make new ribosomes. It appears that what the authors call a ribosome checkpoint registers ribosome biogenesis and relays a signal to the cell-cycle machinery to proceed if the cell has exhibited the capacity to make new ribosomes, even though it has 'old' but functional ribosomes at its disposal that it can use to grow. If this putative checkpoint is a general-wide component of cells, then conditionally deleting *nucleolin* in any dividing cell type like fibroblasts might activate the checkpoint (In this experiment assaying for progress through G1 would begin as soon as previously-synthesized Nucleolin had degraded so that cells no longer had any Nucleolin left after removing *nucleolin* [And this strategy assumes that old ribosomes will not similarly decay at the same rate, so that there are enough to support growth but not proliferation.].). If cells were to proceed through G1 following *nucleolin* deletion, this result could be interpreted to mean that a redundant factor exists to support new ribosome biogenesis. Even if such a redundant factor could compensate for Nucleolin in its absence, Nucleolin might nevertheless be required specifically for the purpose of de-activating the checkpoint; the checkpoint might register ribosome biogenesis that is coordinated with the cell cycle through a particular step that is uniquely effected by Nucleolin. While cell growth that is uncoupled from the cell cycle might be promoted by

both Nucleolin and the hypothetical redundant factor, growth that is coordinated with cell division might be the exclusive domain of Nucleolin. In a cell like post-mitotic neuroblasts, conditionally deleting *nucleolin* might have no effect since these cells might be able to use old ribosomes to make proteins for dendrites and an axon or to use new ones whose assembly is assisted by the redundant factor.

If Nucleolin were under the control of the regulatory mechanism that coordinates cell growth and cell division, this control might be similar, if the not the same, in dividing fibroblasts and dividing neuronal precursors. Such regulation would be removed when neuroblasts exit the proliferative phase of their development. Growth is still required in post-mitotic neuroblasts to support axon and dendrite formation, and so Nucleolin might still be used in its capacity to assist making ribosomes. It would be interesting to understand what its core activities may be in neuronal growth and how they would proceed despite the removal of regulatory modifications that made its function contingent on the cell-cycle machinery. Nucleolin is phosphorylated by cdk1, $p34^{cdc2}$ (Belenguer et al 1990), and Cyclin E- and Cyclin A-associated cdk2 (Sarcevic et al 1997). The consequences of phosphorylation by the latter two have not been investigated since its discovery. Phosphorylation by the former appears to localize Nucleolin during mitosis. However, the helicase activity of Nucleolin is enhanced as well, in which case it would be important to pinpoint the purpose to which it is directed.

Since cell growth appears to be dominant over cell proliferation, it may be that the coordinating device operates downstream of Nucleolin function. Therefore, ribosome biogenesis and Nucleolin activity may depend more on the extracellular environment (assuming a normal intracellular milieu with no checkpoints activated), like growth-factor supply, and the cell-cycle machinery may follow when the cell senses that conditions are suited to growth and division. In this case Nucleolin might actually assist in coordinating cell growth and the cell cycle. A cell that is meant to divide might use Nucleolin after it has participated in ribosome biogenesis as a signal to the cell cycle machinery and possibly as a direct participant in cell proliferation. In a slight variation to this scenario, a different pool of Nucleolin may be used for this purpose, activated when the cell registers an adequate amount of ribosomes.

Questions of high priority in the Nucleolin field must concern Nucleolin sub-fragments and their functionality. No work has addressed whether or not there are mRNA splice variants leading to active Nucleolin segments. Another pathway through which segments may arise is the use of internal sites of translation initiation on Nucleolin mRNA or premature translational termination. There is no evidence to suggest these events occur, although this does not preclude their possibility. Nucleolin fragmentation has been documented (Chen et al 1991; Warrener and Petryshyn 1991), and it is imperative that knowledge be gathered about the possible activity of the fragments that are produced. Results from different Nucleolin studies suggest that Nucleolin segments may have an effect that is different from that of whole Nucleolin. For example, while whole Nucleolin might de-condense chromatin, the N-terminus, after it is phosphorylated, might assist phosphorylated H1 in condensing DNA (Erard et al 1988; Erard et al 1990; Kharrat et al 1991). The C-terminus by itself is sufficient to unstack bases within RNA secondary structures (Ghisolfi et al 1992a), and yet the segment encompassing the central and C-terminal regions can anneal complementary RNA base-pair sequences to produce RNA secondary structures (Ghisolfi et al 1992b; Hanakahi et al 2000). Also of interest is whether or not whole Nucleolin interacts with various putative Nucleolin segments in Nucleolin-specific activities. As a hypothetical scenario, in a step-wise fashion in an activity like ribosome biogenesis, whole Nucleolin and Nucleolin segments might cooperate to complete a portion of ribosome assembly. Whole Nucleolin might begin the process by binding rRNA. That in turn might alter its conformation so that it could interact more strongly with a segment like the C-terminus, which, having been brought in proximity to an RNA secondary structure, could unwind it and make the participatory base pairs accessible. A second whole Nucleolin molecule might then bind a short stretch of the exposed sequence, bringing with it a Nucleolin segment containing the central and C-terminal regions (p50). rRNA binding by the latter might have two consequences: 1 p50 might produce an RNA secondary structure whose formation had been preempted by the first secondary structure, both structures being mutually exclusive because of their overlapping sequences; 2 a conformation change in the second whole Nucleolin molecule that is conducive to protein binding to its N-terminus might allow for the recruitment of a protein that is critical to ribosome

assembly. That protein might have enzymatic activity, completing this hypothetical sequence of steps by modifying the rRNA, or it might be a ribosomal protein, requiring the secondary structure constructed by p50 as a binding site, which, now having this structure in sight, it can interact with.

One conspicuously neglected area of Nucleolin research is the regulation of its synthesis. Growth factors that lead to transcription upregulation (and possibly downregulation) of the *nucleolin* gene are unknown, and the *nucleolin* promoter is a black box. It would be interesting to see whether or not the same factors that stimulate rRNA synthesis do so for Nucleolin mRNA as well. Another facet of this area that requires studying is the metabolism of Nucleolin mRNA: possible variations in its stability under different conditions; determining factors regulating that stability. Correlative evidence has been amassed to show that Nucleolin is synthesized as cells begin to proliferate. However, it is unknown whether Nucleolin synthesis is a precondition for entry into the proliferative phase or it is one of many activities in the immediate wake of cell stimulation by the environment. A cell system in which *nucleolin* can be conditionally deleted might be useful to answer this question.

It is difficult to fully appreciate results from different studies in the Nucleolin field because of the different systems that are used. Also, hints of various activities are presented but not further elucidated. For example, various studies correlate a change in Nucleolin synthesis or activity with cell entry into or departure from the cell cycle. Investigators have observed this using hepatocytes following hepatectomy (Ohmori et al 1990; Konishi et al 1995), peripheral blood mononuclear cells stimulated by phorbol acetate (Westmark and Malter 2001), splenocytes stimulated by LPS (Miranda et al 1995), lymphocytes stimulated by mitogens (Mehes and Pajor 1995), previously serum-starved HeLa cells that have been serum-rescued (Konishi et al 1995), neuroblastoma cells that are differentiating and have exited the cell cycle (Murakami et al 1991) – to give some examples. However, no set of studies has successfully and systematically managed to discern a signaling pathway from growth factor down to transcription factor(s) and co-factors using a single cell type, or related types, and using the same means of growth stimulation. A pathway of this sort would be useful, not only for future experimentation exploiting this information, but also as a reference point while

employing different systems to examine the relationship between Nucleolin activity and the cell- and growth-cycle. A systematic examination of the *nucleolin* enhancer/promoter region alone would offer insights into developmental regulation of its expression (tissue-specificity and possibly timing of expression) and into cellular responses to different extracellular (and intracellular) conditions in the form of a pathway(s) leading to the *nucleolin* gene.

Concerning the second point, there have been three studies that focused on Nucleolin-mediated chromatin re-configuration, with no follow-up since these results were presented a decade ago. Scant work has been devoted to Nucleolin activity in assisting to produce and disassemble RNA secondary structures. There are studies to show that Nucleolin can be a transcription repressor, a helicase, an RNA-binding protein affecting mRNA stability and localization, and an interacting partner with different proteins, ribosomal and non-ribosomal. However, there has been no real attempt to relate these various activities to a specific biology, or, for that matter, to understand what the different activities may have in common. Are all the interactions that Nucleolin has with ribosomal proteins RNA dependent? Growth promotion is deduced for its various nucleolar activities since the nucleolus is the assembly site of ribosomes that the cell requires for growth. It may be useful to understand how and why a characteristic activity of Nucleolin was evolutionarily 'borrowed' for use in a biological function that is distantly related to growth. This approach may in turn provide a means of predicting other biological functions in which Nucleolin might turn up. As an example, its helicase activity might be of service during DNA repair as well as in ribosome biogenesis, especially repair of damage that may occur during mitosis, for which Nucleolin might be particularly suited in this capacity since a high amount of Nucleolin may already be available, being synthesized during the early part of G1 as cells attempt to accumulate enough mass to support another round of the cell cycle. Precise knowledge of how Nucleolin functions in a non-growth-related activity might, in turn, provide insight into how it functions in ribosome biogenesis. Such knowledge may form the basis for experimental design work to clear up the mystery of the exact steps in ribosome biogenesis where Nucleolin functions and how it acts at those steps. For example, even if nothing were known of its functions in ribosome assembly, an accurate depiction of

Nucleolin action in immunoglobulin switch recombination might provide a good starting point for investigating its role in ribosome biogenesis: during switch recombination Nucleolin interacts with hnRNP D to form the LR-1 complex (Dempsey et al 1999), and this finding alone might suggest Nucleolin self-association, given its similarity to the hnRNP class of proteins (see below); as LR-1 can bind G-rich DNA single and double strands, it might be predicted that Nucleolin alone or as a homodimer might interact with G-rich segments of rRNA that is either in a single-strand conformation or in duplex secondary structure.

If Nucleolin could be considered more as an hnRNP protein factor, then experimental strategies and thinking within the hnRNP world might lend themselves to this alteration in perspective concerning Nucleolin function (Dreyfuss et al 2002). Like Nucleolin, hnRNPs generally have modular structure, with RNA-binding and protein-interacting domains (see review in Dreyfuss et al 2002). These factors have been thought to participate exclusively in one activity, the binding and alteration of pre-mRNA transcripts (hnRNAs) within the nucleus, working separately from splicing and export factors (Dreyfuss et al 2002). However, with the discovery that hnRNP factors can shuttle to the cytosol, along with splicing factors, and that some can remain with mRNAs to form different mRNP particles, old distinctions between various RNA-interacting proteins are being abandoned. Nucleolin does interact with at least two proteins that are strictly classified as hnRNP factors, hnRNP C (Zaidi and Malter 1995) and hnRNP D (Dempsey et al 1998). With the former Nucleolin binds the Amyloid Protein Precursor RNA, and with the latter, it binds G-rich DNA. It may turn out that Nucleolin participation in other hnRNP-related activities is more widespread. Studying hnRNP factors with Nucleolin in mind and discerning those particular characteristics of Nucleolin that are specific to Nucleolin only may together be reciprocally beneficial to both areas of investigation. What role, if any, does Nucleolin have in splicing? In export of protein complexes out of the nucleus – does it interact with nuclear core proteins and nuclear transport receptors? Is its interaction with mRNA a more general phenomenon than previously thought? What proteins can hnRNP factors interact with independently of RNA binding, the way Nucleolin interacts with non-ribosomal proteins in an RNA-independent manner (in contrast to RNA-dependent binding to ribosomal proteins)?

It was implied earlier that a standardization of sorts should be undertaken regarding cell types and methods of study to simplify understanding of Nucleolin function. However, given the multifunctional nature of Nucleolin, it may be just as useful to exploit different cell types that vary according to their specific biological needs and functions to fully elucidate each of the proposed activities of Nucleolin. As an example, Nucleolin is proposed to inhibit DNA replication as part of a heat-shock response (Wang et al 2001), and use of a melanoma line to study heat-shock was deliberate since dermal cells, though shielded by the epidermis, are somewhat exposed to sunlight and heat and so would be expected to have fine-tuned a response to heat. If use of Nucleolin in this pathway were to represent an evolutionary exploitation of an already understood activity of Nucleolin – i.e., DNA binding, helicase activity – then this system could not illustrate a new principle of Nucleolin function. However, if a previously undocumented activity of Nucleolin were uncovered, then this system could be exploited to provide more details. Other systems might, in turn, allow for a broader perspective. When the body is in fever and internal cells that are normally not subject to heat rise in their temperature, is Nucleolin activated to facilitate a response? If so, is Nucleolin used in the same way in all responses, or are there cell type-specific variations in Nucleolin employment? Another point of interest arises from dermal-cell proliferation: dermal cells must coordinate the uses of Nucleolin in ribosome biogenesis and heat-shock response. Were the Nucleolin molecules that inhibit Replication Protein A (RPA) previously devoted to ribosome biogenesis during cell division? The Nucleolin localization data from Wang *et al*, indicates that this is the case. However, it brings to mind further questions concerning where Nucleolin is directed to when it is no longer being used for ribosome biogenesis and how and why this re-localization is conducted.

To elaborate on this point further, a cell that undergoes massive cell growth during a post-mitotic phase, like the sciatic nerve, might be a good system to study Nucleolin purely as a ribosome-biogenesis factor. As the cell has exited the cell cycle, whatever influences the cell-cycle machinery may have on Nucleolin can be eliminated as a variable. Also, this system might be particularly well-suited to study another aspect of Nucleolin biology: Nucleolin has been proposed to act as a nucleocytoplasmic shuttling factor (Peter et al 1990), bringing into the nucleus ribosomal proteins and exporting into

the cytosol ribosomal subunits, and its export ability might be especially acute in neurons where functional ribosomes are found in dendrites and the synaptic region of the axon that is far from the nucleus.

In B cells, what is the pre-condition to Nucleolin function at heavy chain switch regions? Since Nucleolin does not interact with this genomic region in other cell types, it is assumed that requisite activities have already occurred to optimize its binding to the G-rich switch regions in B cells. How is that particular binding coordinated with Nucleolin binding to G-rich sequences of rDNA?

A good system to study other Nucleolin activities that serve no growth purpose might be a post-mitotic cell type that devotes a large store of its metabolic energy to a task that is characteristic of its differentiated phenotype. Although such a cell must continue to make ribosomes to survive, it no longer needs to accumulate mass to the same extent as a dividing cell. Therefore, any particular Nucleolin activity that is separate from its role in ribosome biogenesis might be more emphasized in this case.

The primary approach to Nucleolin work has been biochemical in nature. A genetic approach has been relatively neglected. No mammalian genetic reagent exists, like a *nucleolin*-knockout mouse or cells with the endogenous gene deleted. Knockouts of the *S cerevisiae* and *S pombe* orthologues (*NSR1* in the former, *gar1* in the latter) show both a defect in ribosome biogenesis and a slow-growth phenotype (Girard et al 1992; Gulli et al 1995; Kondo and Inouye 1992). Since Gar1p can rescue *nsr*-null cells, at least in the capacity of ribosome-subunit production, it can be concluded that the two proteins are functionally equivalent with regards to ribosome biogenesis. However, there are indications that mammalian and yeast Nucleolin are distinct: 1 although rDNA transcription appeared to be unaffected in the yeast knockouts, suggesting that yeast Nucleolin does not participate in this step of ribosome biogenesis, available evidence indicate such participation on the part of mammalian Nucleolin; 2 mammalian Nucleolin aids in cleaving a site in the 5' ETS of the pre-rRNA transcript, and yet, considering that all rRNA transcripts besides the 18S rRNA transcript were produced normally in the yeast knockouts, the conclusion to be drawn is either that producing these transcripts is independent of this processing reaction or that processing is unnecessary for yeast rRNA maturation; 3 hamster Nucleolin does not functionally complement the *nsr*-null strain.

Nevertheless, the absence of the 40S subunit in the yeast knockout strains strongly indicates some sort of role in ribosome biogenesis for both Nucleolin orthologues, and an attempt to uncover the specific steps of ribosome biogenesis in which they participate may be warranted to understand mammalian Nucleolin function better. With this mind, one possible starting point would be a more systematic deletion and point-mutation analysis of Gar1p in its capacity as ribosome-biogenesis factor (The *S pombe* orthologue is chosen to initiate these studies since *S pombe* appears to have no redundant function that would complicate interpretation of results.). What would be the consequence of deleting the region that is equivalent to the N-terminus of mammalian Nucleolin? If this genetic manipulation were to cause a defect in ribosome assembly, could the resulting immature ribosome particle or particles be isolated for analysis? If so, could its components be determined? A comparison of an immature ribosomal particle from the mutant with the same particle (isolated in the same way and from cells under the same conditions and during the same stage of the cell cycle) from the wild-type strain might reveal a deficit in certain ribosomal proteins and an improper rRNA structure in the former particle.

One new approach in ribosome research is to attain ribosomal particles by isolating a component in such a way that its association with rRNA and other proteins, ribosomal and non-ribosomal, is preserved. Therefore, in purifying the component under different cell conditions and different cell-cycle stages, immature ribosomal particles of which it is part are also isolated. Two groups used this strategy focusing on Nucleolin. In one case Nucleolin was directly immunoprecipitated (Piñol-Roma 1999) and in the other a tagged Nucleolin was immunoprecipitated through the use of the tag (Yanagida et al 2001). In two other studies (Hampichamchai et al 2001; Bassler et al 2001) ribosomal particles from yeast were isolated using the tandem affinity purification (TAP) protocol (Rigaut et al 1999), in which tagged Nop7p and Nug1p (two ribosomal components) were the targets of isolation. As Jonathan Warner suggests in his review on this methodology (Warner 2001), these particles are useful not only for their content but also as a starting point for further analysis of other stages of ribosome biogenesis. A component that is found in one particle can itself be used as a means of ribosome purification. Complexes isolated in this way may represent subsequent stages in ribosome assembly and

themselves may contain components whose purification will lead to other previously unknown immature complexes. Even if by protein composition two pre-ribosomal particles appear to be the same, there may be subtle differences that point to one particle as representative of a later step in ribosome assembly. Hypothetically speaking, two such Nucleolin-containing particles may both possess the same proteins (and maybe in the same stoichiometric relations) and the same number of Nucleolin molecules and segments, but closer examination (UV cross-linking combined with crystallography) of both particles may show that while in one particle rRNA-bound Nucleolin interacts with a Nucleolin segment (C-terminus: GAR domain [Gd]) as a result of a conformation change upon rRNA binding, in the other particle Gd is separated from whole Nucleolin and is now bound to a protein that sits in a nearby region of the same rRNA – Gd may have disassembled a secondary structure that altered the rRNA in such a way that the region where the protein resides was brought into proximity of the Nucleolin-Gd pair; the affinity of Gd for the protein is higher than that for whole Nucleolin, and therefore Gd interacts with the protein as it loses its grip on whole Nucleolin – the transition between these two particles may be too quick to isolate an intermediate particle, one in which the secondary structure is midway towards disassembly or one in which disassembly is complete but the binding between Gd and the ribosomal protein has not yet been initiated.

As mentioned, past studies to elucidate Nucleolin function utilized biochemistry, whether through extracts or through cultured cells. However, a combined approach utilizing this type of work, as exemplified by Bouvet's group in France (that is largely responsible for demonstrating Nucleolin involvement in rRNA processing in the 5' ETS), pre-ribosome particle isolation, and genetics could go a long way towards hastening the work to construct the picture of Nucleolin-assisted ribosome biogenesis. The following is once again hypothetical, presented to illustrate the point just made. Isolation of a mammalian pre-rRNA processing (not necessarily the 5' ETS cleavage) complex may reveal a protein, X, as a component. An orthologue of X has been located in *S cerevisiae* and been shown to be functionally similar to its mammalian counterpart. As a result, deletion of the gene encoding X is undertaken, with the result that processing is interrupted in the mutant. An isolated processing complex turns out to be different in

composition to the mammalian complex in which X was originally observed. Genes for all components of the complex from the mutant yeast strain have been cloned already, and the components can be readily purified. In an *in vitro* assembly reaction, the different components are mixed together in order-of-addition experiments to determine whether or not formation of the rRNA-processing complex can be duplicated cell-free. The experiment works, and genetic confirmation is next attempted. By deleting the gene for each component and then isolating the processing complex from each mutant, it is found that the steps of the processing reaction exactly mirror the order in which each component comes into play in the *in vitro* processing reaction. The point of this exercise is to show that further ground can be gained in determining Nucleolin function if different approaches are combined. A purely genetic approach, as in use of epistatic interactions, could provide a sketch of a pathway in ribosome biogenesis (i.e., activity of W depends on O activity since O deletion renders W activity unnecessary); however, it cannot provide the kind of detailed information that biochemical assays can (i.e., O binds the pre-ribosome and alters the structure that allows for recruitment and insertion of W into particle); while a purely biochemical strategy would entail having to purify particles and components and determining interactions 'two protein factors at a time' and particle assembly step-by-step, a strategy involving genetic interactions, as just indicated, could provide a quick outline of a pathway whose details could then be acquired with the precision afforded through biochemistry. Both approaches complement and reinforce each other. Particle isolation by itself suffers from disadvantages peculiar to this approach, the inability to isolate transitory structures, the laborious nature of scrutinizing each particle through difficult techniques like crystallography.

A future area of Nucleolin work will be to determine its role in other rRNA processing reactions, like the kind that directly produce the rRNA transcripts of the ribosome subunits. A three-pronged strategy like the kind described above could accelerate data acquisition to reach a fuller understanding of Nucleolin function in this particular aspect of ribosome biogenesis.

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This discussion has attempted to offer suggestions on how to consolidate past findings on Nucleolin activity and ways to more quickly reach a deeper understanding of its biology.

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